

## CHARACTERIZATION OF FREE NITROGEN FIXING BACTERIA OF THE GENUS *Azotobacter* IN ORGANIC VEGETABLE-GROWN COLOMBIAN SOILS

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Submitted: June 18, 2010; Returned to authors for corrections: January 13, 2011; Approved: March 14, 2011.

### ABSTRACT

With the purpose of isolating and characterizing free nitrogen fixing bacteria (FNFB) of the genus *Azotobacter*, soil samples were collected randomly from different vegetable organic cultures with neutral pH in different zones of Boyacá-Colombia. Isolations were done in selective free nitrogen Ashby-Sucrose agar obtaining a recovery of 40%. Twenty four isolates were evaluated for colony and cellular morphology, pigment production and metabolic activities. Molecular characterization was carried out using amplified ribosomal DNA restriction analysis (ARDRA). After digestion of 16S rDNA Y1-Y3 PCR products (1487pb) with *AluI*, *HpaII* and *RsaI* endonucleases, a polymorphism of 16% was obtained. Cluster analysis showed three main groups based on DNA fingerprints. Comparison between ribotypes generated by isolates and *in silico* restriction of 16S rDNA partial sequences with same restriction enzymes was done with Gen Workbench v.2.2.4 software. Nevertheless, Y1-Y2 PCR products were analysed using BLASTn. Isolate C5T from tomato (*Lycopersicon esculentum*) grown soils presented the same *in silico* restriction patterns with *A. chroococcum* (AY353708) and 99% of similarity with the same sequence. Isolate C5CO from cauliflower (*Brassica oleracea* var. *botrytis*) grown soils showed black pigmentation in Ashby-Benzoate agar and high similarity (91%) with *A. nigricans* (AB175651) sequence. In this work we demonstrated the utility of molecular techniques and bioinformatics tools as a support to conventional techniques in characterization of the genus *Azotobacter* from vegetable-grown soils.

**Key words:** ARDRA; *Azotobacter*; FNFB; UPGMA.

### INTRODUCTION

The genus *Azotobacter* belongs to the  $\gamma$ -subclass of the *Proteobacteria* (4, 34) and comprises seven species: *A. chroococcum*, *A. vinelandii*, *A. beijerinckii*, *A. paspali*, *A. armeniacus*, *A. nigricans* and *A. salinestri*. In 2005 sequences

of 16S rDNA were identified that corresponded to a new endemic soil species from Thailand (unpublished data). *Azotobacter* are heterotrophic and aerobic bacteria and their main property is the ability to fix nitrogen non-symbiotically, with a genomic content of G-C of 63-67.5 % ( $T_m$ ) (4, 32), and distributed in soils, water and sediments (35, 36). Their

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agronomic importance is due to the capability of synthesizing antibiotics, plant growth promotion substances (21, 24, 25, 30), vitamins, exopolysaccharides and pigment production (8, 28), besides their antagonist effect against pathogens (33). They also have the ability to solubilize phosphates in aquaculture systems, vermicompost production and potato (*Solanum tuberosum*) grown soils (9, 12, 19). In Colombia, it has been used as bacterial inoculants in the culture of *Dendranthema grandiflora*, *Stevia rebaudiana*, vegetables and cotton (6, 11, 29). Characterization of *Azotobacter* species becomes difficult due to the morphological similarity that exists with other FNFB like *Derxia*, *Azomonas* and *Beijerinckia*, in addition to its interspecific biochemical ambiguity. For that reason, in the last years authors like Aquilanti *et al.* (1, 2), and Becking (4), among others, justify the use of molecular techniques and bioinformatics tools allowing fast identification and characterization. This interest is focused towards the validation of fast and reliable molecular methodologies for quality control on agricultural biofertilizers. In this context, amplified ribosomal DNA restriction analysis (ARDRA) was evaluated for the characterization of *Azotobacter* isolated from organic vegetable-grown soils, with the purpose of generating a molecular approach to this genus in Colombian agroecosystems.

## MATERIALS AND METHODS

### Isolation and biochemical characterization

Soil samples from organic broccoli (*Brassica oleracea* var. *italica*), zucchini (*Cucurbita pepo*), cauliflower (*B. oleracea* var. *botrytis*), spinach (*Spinacia oleracea*), tomato (*Lycopersicon esculentum*) and carrot (*Daucus carota*) grown soils were collected during October 2006 in Sogamoso (5°43'N, 72°55'W) and Tibasosa (5°44'N, 73°00'W) (Boyacá-Colombia) (Table 1). Five random samples each of 500 g were withdrawn from 10–15 cm depth and sieved through a 4.75 mm-mesh sieve. Soil pH was measured according to Van

Lierop (38), analyzing samples fully suspended in distilled water 1:1 (v/v). The primary isolation was made using the grain soil technique (Figure 1A) according to the methodology previously described by Aquilanti *et al.* (1) and Becking (4) in Ashby-Sucrose agar (Agar 1.5%, Sucrose 0.5%, CaCO<sub>3</sub> 0.5%, MgSO<sub>4</sub> 0.02%, NaCl 0.02%, KH<sub>2</sub>PO<sub>4</sub> 0.02%, FeSO<sub>4</sub> 0.0005%). Plates were incubated at 28°C for 7 days until observing sticky and glistening colonies around grains (Figure 1F). Counts were made for each cultivated soil (% Recovery = Number of grains with sticky and glistening colonies/total x 100). Isolates were purified by streaking on free nitrogen Ashby-Sucrose agar and morphological observations were done and subsequently stored at -80°C in nutrient broth (Scharlau®, Barcelona, Spain) containing 50% glycerol. Pigment production was observed by growing the cultures in Ashby agar with 0.5% (w/v) of Benzoate (4, 22) and incubation at 28°C for 7 days. *A. vinelandii* ATCC12518 and *Azotobacter* sp. strain (CCT) previously isolated were used for biochemical identification and molecular characterization. Biochemical identification was carried out in triplicate using different sources of carbon: glucose (GLU), maltose (MAL), mannitol (MAN), rambiose (RAM) 1% (w/v) and benzoate (BNZ) 0.5% (w/v) in phenol red broth (Merck®, Germany). Oxidase (OXI), catalase (CAT) and nitrate reduction tests were done (34) and biochemical sets were incubated at 28°C and evaluated after 24 h.

### Genomic DNA extraction

Cultures were grown overnight in 5 ml nutrient broth, shaken at 150 rpm and 20°C for 48 h. A 1.5 ml of the culture was centrifuged at 10 000 g for 2 min and the resultant pellet was resuspended in 1050 µl 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA) and centrifuged at 10 000 g for 2 min, resuspended again in 350 µl 1X TE buffer, 5 µl lysozyme (50 mg/ml) and 2 µl RNase A (10 mg/ml) (Sigma®, St Louis, USA), and incubated at 37°C for 10 min. Finally, 3 µl proteinase K (20 mg/ml) (Invitrogen®, Steinheim, Germany) and 30 µl SDS 10% were added and incubated at 65°C for 15

min. Crude DNA template was PCI (Phenol - Chloroform - Isoamyl alcohol) (25:24:1) and CI (Chloroform - Isoamyl alcohol) (24:1) extracted, precipitated in 1 ml absolute ethanol and washed with 500 µl 70% ethanol and resuspended in 50 µl of sterilized ultrapure water and stored at -20°C.

### ARDRA

A 1487 bp fragment was amplified from the 16S rDNA gene using universal primers Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and Y3 (5'-TACCTTGTTACGACTTCACCCAGTC-3'). (20, 40). PCR reaction was done in a 100 µl reaction mixture containing: 1 U of Taq DNA polymerase (Biolase®, Luckenwalde, Germany), 1X NH<sub>4</sub> reaction buffer (160 mM (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20), 2 mM of MgCl<sub>2</sub>, 50 µM of dNTPs, 0.2 µM of each primer and 50-100 ng of DNA. PCR reactions were run for 35 cycles on a My cyclor (Biorad®, Hercules, CA, USA) as follows: denaturation at 93°C for 45 s, annealing at 62°C for 45 s, and elongation at 72°C for 2 min. An initial denaturation step at 93°C for 2 min and a final extension step at 72°C for 5 min (16) were also done. DNA amplification was checked by electrophoresis of 10 µl of each PCR product in a 1.0% (w/v) agarose gel, in 1X TBE buffer (0.09M Tris base, 0.09M sodium borate, 2.5 mM EDTA, pH 8.3) for 1 h at 3.2 V/cm on a Primo Thermo EC 330 Midcell (Waltham®, MA, USA). A 25 µl-aliquot of the PCR product was digested in a final volume of 40 µl for 3 h at 37°C with 2.5 U of restriction enzyme (*AluI*, *HpaII* or *RsaI*), 1X reaction buffer, and 0.1 mg/µl of BSA, according to the manufacturer (Promega®, Madison, USA). PCR products digests were separated in a 2.5% (w/v) agarose gel electrophoresis in 1X TBE buffer for 3 h at 3.2 V/cm and gels were stained in ethidium bromide 0.5 µg/ml. DNA fragments were visualized at 312 nm with an UV-transilluminator. Gels electronic images were captured and analysed in the Quantity One 1-D Analysis software (Biorad®, Hercules, CA, USA). For data scoring only 50 bp fragments or highest were considered to binary data

matrix built. Molecular weights of each band were determined by means of a linear regression analysis, having as reference a 100 bp ladder (Promega®, Madison, USA). Genetic distance between isolates was calculated using the DICE coefficient of similarity and resulting distance matrix was used for the unweighted pair group method with arithmetic mean (UPGMA) analysis using NTSYSpc v.2.2 software for Windows (27). The consistency of each node was estimated by bootstrapping across markers (10) using 1000 pseudoreplications. *In silico* restriction analysis was carried out with *AluI*, *HpaII* and *RsaI* using partial sequences of 16S rDNA obtained from the following GenBank accessions: AAY336565 (*A. vinelandii*); AY353708 (*A. chroococcum*); AJ308318 (*A. paspali*); AB175656 (*A. salinestris*); AB175655 (*A. armeniacus*); E F100152 (*A. beijerinckii*); AB175651 (*A. nigricans*); AF112477 (*Azospirillum*); AB175654 (*Azomonas macrocytogenes*); AJ563934 (*Beijerinckia dexti*); AF164045 (*Burkholderia tropica*); AB089482 (*Derxia gummosa*); AY191275 (*Herbaspirillum seropedicae*); AY509900 (*Rhizobium leguminosarum*) and AB305017 (*Escherichia coli*), using Gene Workbench v.2.2.4 software for Windows® (CLCbio®, Maryland, USA).

### Sequence analyses

Primers Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3') were used for the analysis of a 318 bp partial sequence of 16S rDNA (2). PCR reaction was done in a 100 µl reaction mixture containing: 1 U of Taq DNA polymerase (Biolase®, Luckenwalde, Germany), 1X NH<sub>4</sub> reaction buffer (160mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 670mM Tris-HCl pH 8.8, 0.1% Tween 20), 2 mM of MgCl<sub>2</sub>, 200 µM of dNTPs, 0.5 µM of each primer and 50-100 ng of DNA. PCR reactions were run for 35 cycles on My Cyclor (Biorad®, Hercules, CA, USA) as follows: denaturation at 93°C for 45 s, annealing at 62°C for 45 s, and elongation at 72°C for 2 min. An initial denaturation step at 93°C for 5 min and a final extension step at 72°C for 5 min were also done.

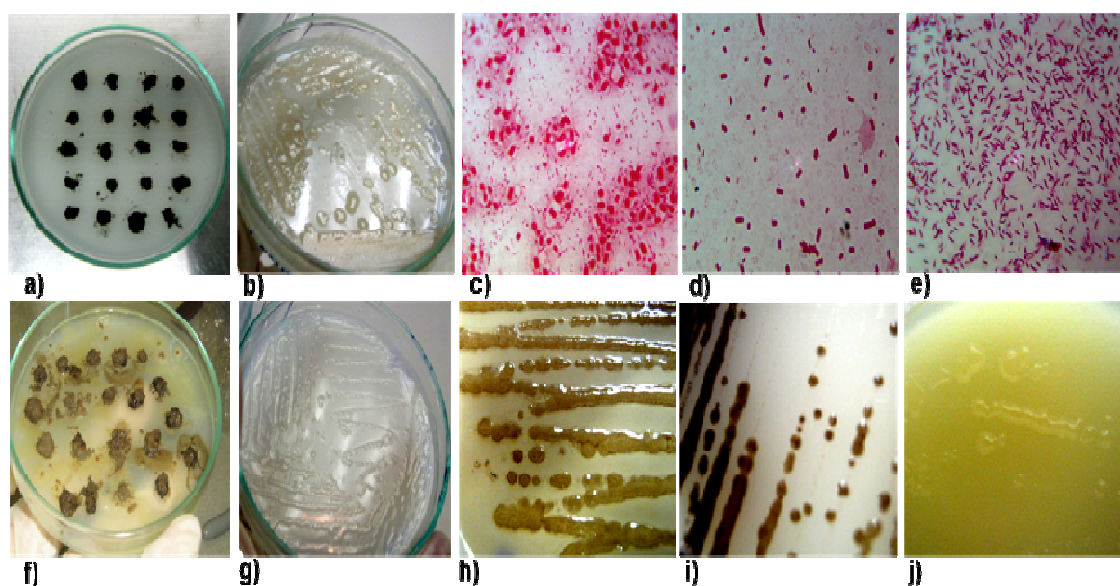
Amplification products were quantified using a Beckman DU 500 spectrophotometer (Fullerton®, CA, USA) and sequenced. Sequences were aligned in Combined Workbench v.3.6.1 software for Windows (CLCbio®, Maryland, USA) and compared with the data bases of GenBank using BLASTn (5).

## RESULTS

### Morphological and biochemical identification

The soil pH is within the range 6.25-7.44, which has been reported optimal for the growth of *Azotobacter* species (3, 4). Those pH values facilitated the recovery, allowing obtainment percentages between 31% and 45% for the crop fields grown with six different crops. A total of twenty-four isolates were obtained. Most isolates presented whitish (cream color), smooth, irregular, shining, 3-8mm diameter colonies; nevertheless, colonies with transparent, glistening, shining, 2-5

mm diameter also appeared. Three cell type morphologies were identified: Gram-negative bacilli short and large; Gram-negative bacilli short and small; and cysts (Figure 1). Some showed a brown pigmentation and others presented a dark brown pigmentation; a single one displayed a yellowish green pigmentation, which is a characteristic of both *A. vinelandii* and *A. paspali* (2, 4). Twelve isolates were glucose fermentation positive, six showed a doubtful result (Table 1), and twenty two were catalase positive. Isolate C5T was ramnose negative; nevertheless it presented a doubtful result for glucose fermentation. For preliminary identification (Table 1), morphological traits (cell and colony morphology) and pigment production in the Ashby-Benzoate agar were considered. Additionally, both control *A. vinelandii* ATCC12518 and CCT strains identities were confirmed with the same test.



**Figure 1.** Isolation, morphological traits and pigment production of nitrogen fixing bacteria isolates from vegetable-grown soils. a) Primary isolation using soil grain technique. b) Whitish (cream color), smooth, irregular, shining, 3-8mm in diameter colonies, isolate C5T. c) Cysts, isolate C5CA. d) Short and large Gram-negative bacilli, isolate C5T. e) Small Gram-negative bacilli isolate C1BR. f) Sticky and glistening colonies growing around soil grains g) Transparent, glistening, shining 2-5mm in diameter colonies, isolate C1BR. h) Brown pigmentation, isolate C1T. i) Brown-black pigmentation, isolate C5T. j) Yellow-green pigmentation, isolate C5CA.

**Table 1.** Crop-grown soil samples, pH, recovery percentage, location, morphological characteristics, biochemical and preliminary identification of nitrogen fixing bacteria isolates from vegetable-grown soils.

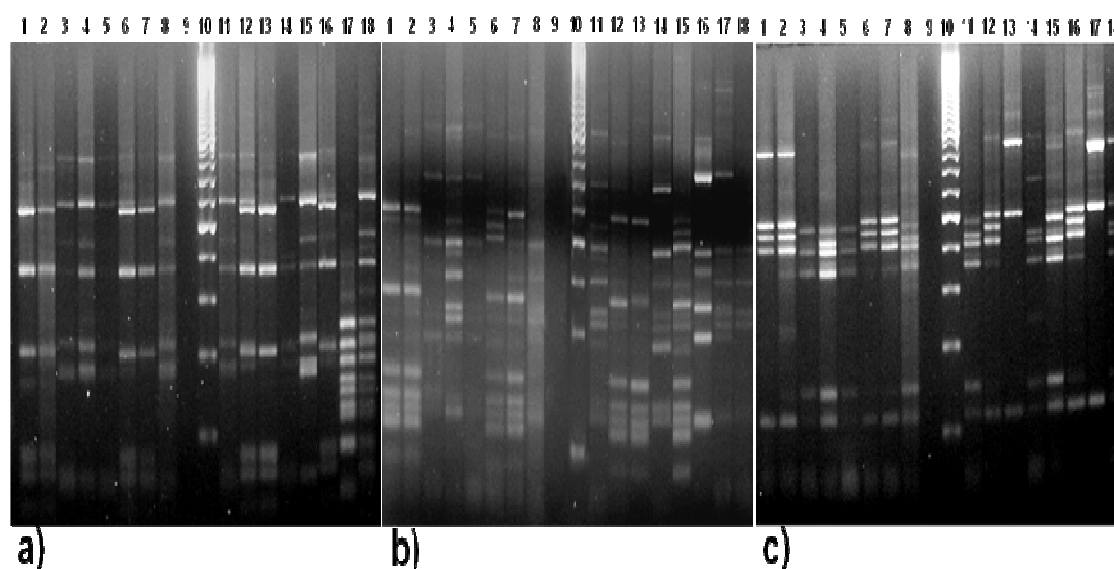
Crop	pH	%R <sup>a</sup>	Location	Isolate	Colony morphology	Cellular morphology	Pigment	GLU	MAL	MAN	RAM	BNZ	OXI	CAT	NITRATE	Preliminary identification	
ND	ND	ND	ND	ATCC 12518	A	Y		+	+	+	+	D	+	+	NITRITE	<i>A. vinelandii</i>	
Potato	ND	ND	ND	CCT				+	+	+	+	+	+	+			+
Broccoli	7.06	36.3	Tibasosa	C1BR	B	Y	NP	-	-	-	-	D	+	-	AMMONIUM	ND	
				C2BR				-	+	+	D	-	-	+			
				C3BR				+	D	+	-	-	+	+	NITRITE	<i>A. nigricans</i>	
				C4BR				X	-	-	-	-	-	+	AMMONIUM	ND	
				C5BR				Y	D	+	+	+	-	+	+	NITRITE	<i>A. vinelandii</i>
Zucchini	6.82	35	Sogamoso	C1CA	A	X	Brown-Yellow	+	+	D	+	+	+	+	AMMONIUM	ND	
				C3CA				-	-	D	-	D	+	+			
				C4CA				D	-	-	-	-	+	+	<i>A. nigricans</i>		
				C5CA				Z	+	+	+	+	+	+	<i>A. vinelandii</i>		
Cauliflower	7.12	33.6	Tibasosa	C1CO	B	Y	NP	Black	D	+	+	-	D	D	+	NITRITE	<i>A. chroococcum/ A. nigricans</i>
				C4CO				D	-	-	-	D	+	+			
				C5CO				+	-	-	D	-	+	+	AMMONIUM	<i>A. nigricans</i>	
				C1E				D	-	-	-	-	+	+			
Spinach	6.79	45	Sogamoso	C2E	A	Y	Brown-Black	+	+	+	+	+	+	+	NITRITE	<i>A. vinelandii</i>	
				C3E				+	+	+	+	+	+	+			
				C4E				X	-	-	-	-	D	+	+	ND	
				C5E				+	D	+	-	D	D	+	AMMONIUM	<i>A. chroococcum/ A. nigricans</i>	
				C1T				Brown	+	-	-	-	-	+	+	<i>A. nigricans</i>	
Tomato	7.44	37.6	Tibasosa	C2T	B	X	NP	-	D	-	-	-	-	+	NITRITE	ND	
				C3T				-	-	-	-	-	+	+			
				C4T				+	+	+	+	+	+	+	NITRITE	<i>A. vinelandii</i>	
				C5T				A	D	+	+	-	D	D	+	AMMONIUM	<i>A. chroococcum/ A. nigricans</i>
Carrot	6.25	31.3	Tibasosa	C1Z	B	Z	NP	+	-	-	-	-	-	+	NITRITE	ND	
				C2Z				-	-	-	-	-	-	+			<i>A. paspali</i>

<sup>a</sup> Recovery percentage; ND: Not determined; A: whitish (cream color), smooth, irregular, shining, 3-8mm in diameter colonies; B: transparent, glistening, shining, 2-5mm in diameter colonies; X: Gram-negative bacillus, short and small; Y: Gram-negative bacillus, short and large; Z: Cysts; NP: Negative pigment; (+): Positive test.; (-): Negative test; D: Doubtful; GLU: Glucose; MAL: Maltose; MAN: Mannitol; RAM: Ramnose; BNZ: Benzoate; OXI: Oxidase test; CAT: Catalase test.

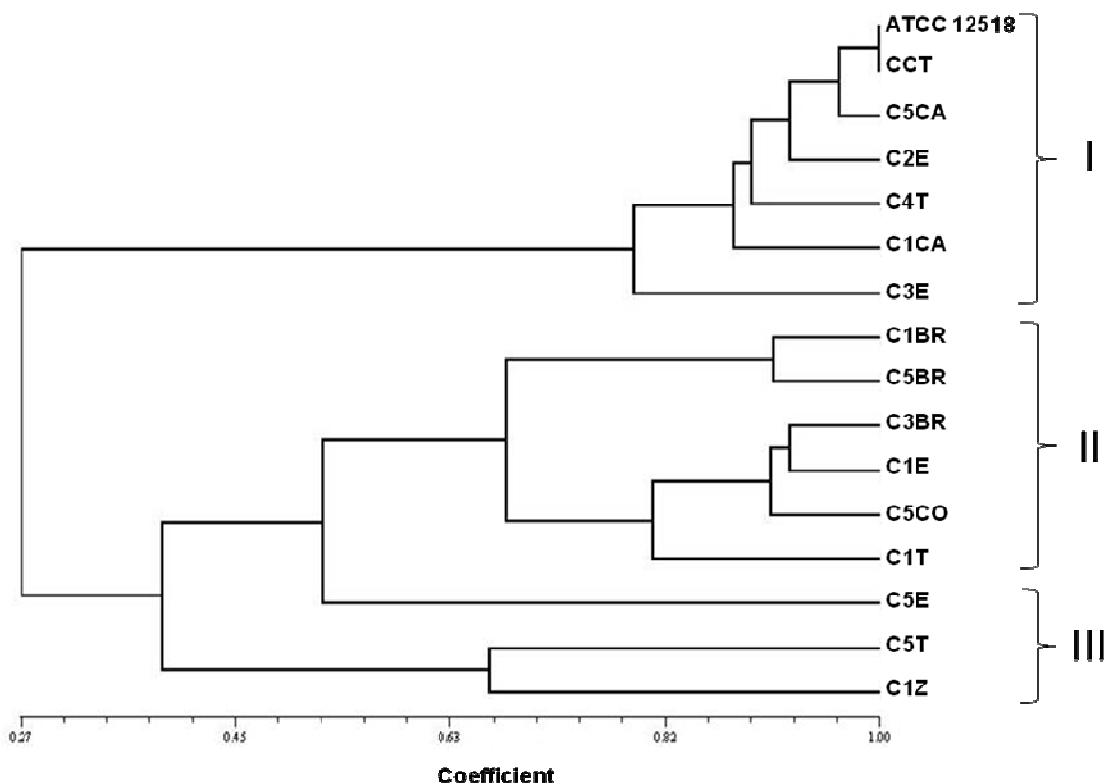
**ARDRA**

Based on the preliminary identification, thirteen presumptive *Azotobacter* isolates (C3BR, C5BR, C1CA, C5CA, C5CO, C1E, C2E, C3E, C5E, C1T, C4T, C5T and C1Z) were selected for DNA extraction and restriction analysis. Isolate C1BR was identified as not belonging to *Azotobacter*, therefore it was included in our study to observe genetic differences with the other isolates that were catalase positive (Table 1). *A. vinelandii* ATCC12518 and CCT strains were included in the molecular analysis. Restriction endonuclease *AluI* generated eight rybotypes, a total of 78 bands and a polymorphism of 16.6%; with *HpaII* twelve rybotypes were obtained, a total of 92 bands and a polymorphism of 17.3%; and *RsaI* generated seven rybotypes, a total of 82 bands and a polymorphism of 15.8% and 1.2% of unique bands (Figure 2). UPGMA showed that the isolates exhibit three main groups (Figure 3). Group I, with a coefficient of similarity of approximately 0.75%. In this group,

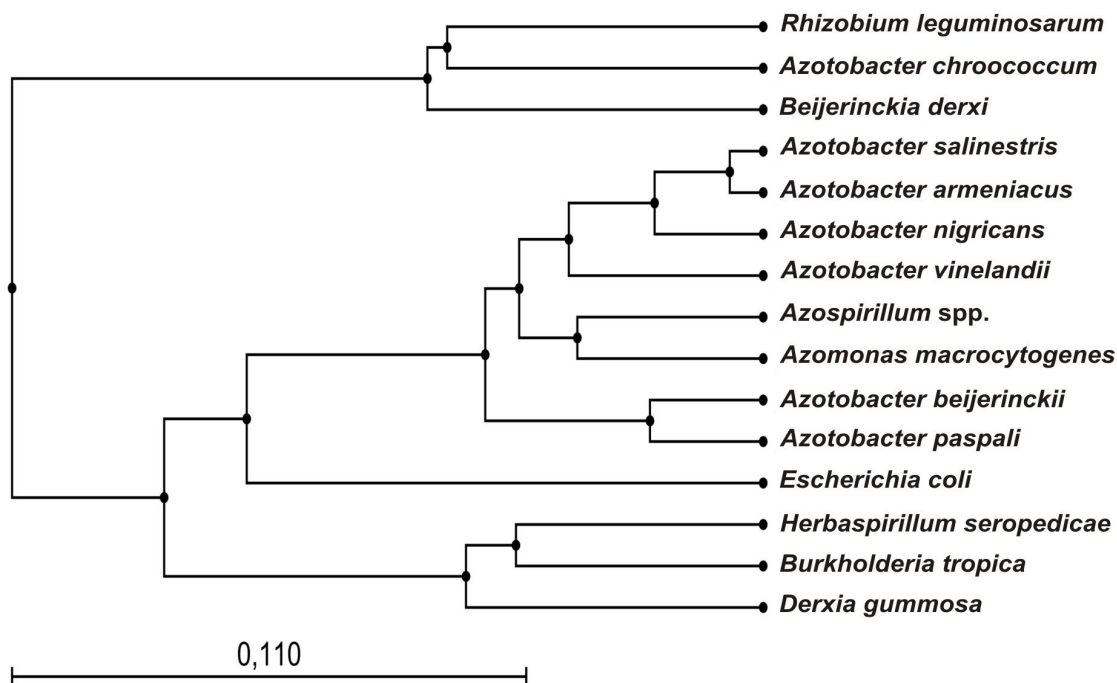
*A. vinelandii* ATCC12518 presented a greater coefficient of similarity (1.0) with respect to CCT strain and a similarity of 0.97 with respect to C5CA. Group II, with a coefficient of similarity of approximately 0.69 includes all the isolates obtained from broccoli-grown soils. Therefore, group III, with a coefficient of similarity of approximately 0.65 includes C5T, C1Z and C3E, the ones that showed the most dissimilar restriction patterns. On the other hand, UPGMA for the *in silico* restriction analysis showed that *Azotobacter* species have similar restriction patterns except for *A. chroococcum* (AY353708) (Figure 4). The *in silico* restriction analysis with *AluI* generated different restriction patterns for all *Azotobacter* sequences and a polymorphism of 48.3%. *HpaII* generated identical restriction patterns for *A. nigricans* (AB175651) and *A. vinelandii* (AY336565) and a polymorphism of 42.4%. Finally *RsaI* generated the same restriction patterns for *A. armeniacus* (AB175655) and *A. salinestris* (AB175656) and a polymorphism of 50%.



**Figure 2.** Agarose 2.5% gel electrophoresis of restriction fragments obtained after the digestion of 16S rDNA amplicons of nitrogen fixing bacteria isolates from vegetable-grown soils. Restriction patterns with: a) *AluI*. b) *HpaII*. c) *RsaI*. 1. ATCC 12518. 2. CCT. 3. C1BR. 4. C3BR. 5. C5BR. 6. C1CA. 7. C5CA. 8. C5CO. 9. Negative control (sterilized ultra pure water). 10. Molecular weight ladder (100pb Promega®). 11. C1E. 12. C2E. 13. C3E. 14. C5E. 15. C1T. 16. C4T. 17. C5T. 18. C1Z.



**Figure 3.** UPGMA for ARDRA of the FNFb isolates from vegetable-grown soils using NTSYSpc v.2.2. software for Windows®.



**Figure 4.** UPGMA for *in silico* restriction of the sequences of FNFb obtained from GenBank database using Gene Workbench® v.3.6.1 software for Windows®.

**Sequence analyses**

The dendrogram obtained after the alignment of partial 16S rDNA sequences (Y1–Y2 primers) was similar to the ARDRA analysis (Figure 5). This result supports the utility of ARDRA for taxonomic classification. Isolates C1BR, C5BR and C1T are included in one group. C3BR and C1E form another group, and C5CO is remote. On the other hand, C5E, C1Z and C5T form individual groups which presented dissimilar restriction patterns in ARDRA. The result of the

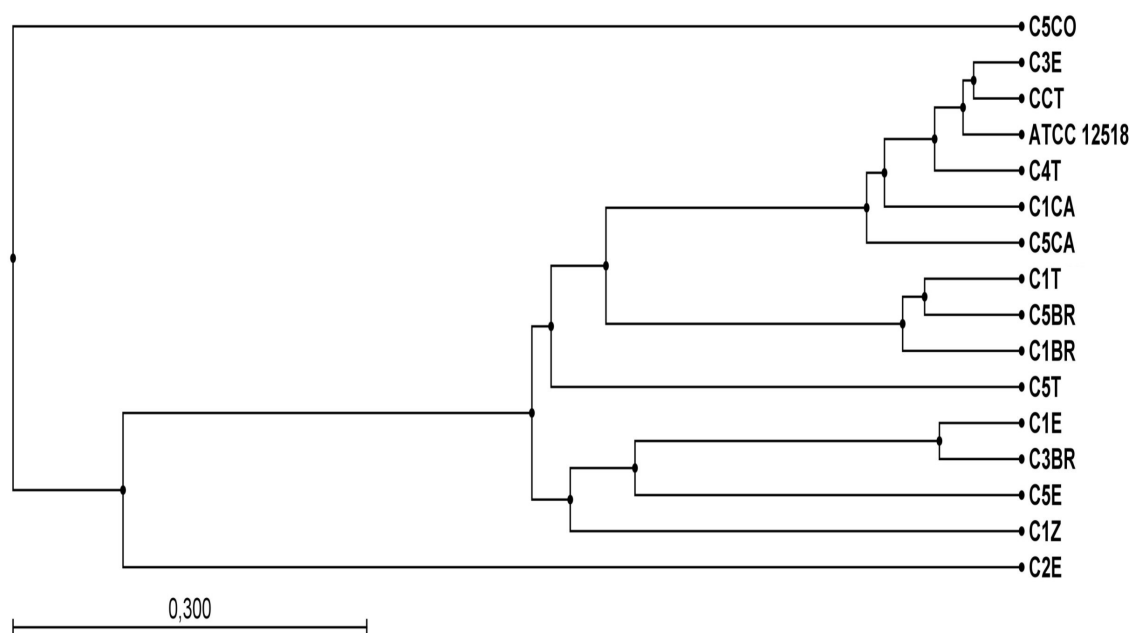
BLASTn (Table 2) showed that the isolates included in group I of ARDRA presented similarity with *A. vinelandii* sequences. With respect to group II of ARDRA, C1BR, C5BR and C1T presented a smaller similarity with *Azotobacter* sequences. Nevertheless, C5T presented a similarity of 99% with *A. chroococcum* (AY353708). The partial 16S rDNA sequences derived in this study have been deposited in GenBank (Table 2).

**Table 2.** Alignment of partial 16S rDNA sequences of FNFB isolates from vegetable-grown soils with sequences from databases (GenBank, EMBL and DDBJ) using BLASTn software.

ARDRA Group	Isolate (Accession number)	Bases pairs	Similarity (%)	Species (Accession number)
I	ATCC12518 (EU935082)	307	79	<i>Azotobacter vinelandii</i> (EF620452)
	CCT (EU935083)	311	84	<i>Azotobacter vinelandii</i> (EF620451)
	C1CA	317	75	<i>Azotobacter vinelandii</i> (EF620439)
	C5CA (EU935087)	317	78	<i>Azotobacter vinelandii</i> (EF620439)
	C2E	318		NS
	C3E	304	84	<i>Azotobacter vinelandii</i> (EF620439)
	C4T	306	80	<i>Azotobacter vinelandii</i> (EF620439)
II	C1BR (EU935086)	318	81	<i>Bacillus cereus</i> (EU368180)
	C3BR	315	89	<i>Clostridium botulinum</i> (L37590)
	C5BR	318	93	<i>Bacillus cereus</i> (AM711591)
	C5CO (EU935085)	320	91	<i>Azotobacter nigricans</i> (AB175651)
	C1E	320	79	<i>Azotobacter</i> sp.(EU784140)
	C1T	318	83	<i>Azotobacter</i> sp. (DQ672579)
III	C5E (EU935088)	314	73	<i>Azotobacter chroococcum</i> (EU647694)
	C5T (EU935084)	312	99	<i>Azotobacter chroococcum</i> (AY353708)
	C1Z	294	79	<i>Azotobacter</i> sp. (DQ672579)

NS: No similarity with any reported sequence





**Figure 5.** UPGMA for aligned sequences obtained with primers Y1-Y2 in each of the FNFB isolates from vegetable-grown soils, using Combined Workbench® v.3.6.1 software for Windows®.

## DISCUSSION

*Azotobacter* species are found in agricultural soils playing different beneficial roles: atmospheric nitrogen fixation, production of phytohormones, degradation of toxic compounds (7, 16) and driving the ecological balance in agro-ecosystems. For the isolation and molecular characterization of FNFB of the genus *Azotobacter* we sampled soils from different organic vegetable cultures located in Boyacá-Colombia, fertilized with considerable amounts of organic matter and phosphates that benefit the proliferation of FNFB as reported by Gordon (13) and Tsain *et al.* (37) and without nitrogen fertilizers in the rotation scheme that could inhibit the growth of *Azotobacter* species (3). FNFB were recovered in free nitrogen Ashby-Sucrose agar (4) and *A. chroococcum* in Ashby agar with mannitol or benzoate (31). Benzoate is used as a carbon source by *A. chroococcum*, which uses the  $\beta$ -ketoacid pathway to convert it into succinate. Succinate is introduced into the respiratory chain by means of the flavine-dependent system

and the production of melanines, which are related to the colony pigmentation (4, 7). In addition, some important microelements such as iron and molybdenum are necessary for nitrogen fixation (18). Carrot-grown soils presented the lowest percentage of FNFB recovery (31.3%), possibly due to its low pH (6.25), while for other soils the recovery was between 35% and 45%. Although most isolates displayed the characteristic colony morphology of the genus *Azotobacter*, some presented transparent, glistening, shining, 2-5mm in diameter colonies, similar to the described ones for other FNFB such as *Beijerinckia* and *Azospirillum* (Figure 1G) (17, 34), which renders colony identification based on morphology a difficult task sometimes producing ambiguous results. Isolates C5CA and C1Z formed the characteristic cysts of *Azotobacter* (Figure 1C). According to Hitchins and Sadoff (15) cysts can be formed by the action of the calcium ion present in the Ashby agar after the exponential phase of growth or under adverse conditions due to the lack of nutrients. These structures are characteristic of *Beijerinckia* although unlike as in *Azotobacter*,

in this case they do not appear forming pairs. An important characteristic of *Azotobacter* is the Gram-negative bacillary morphology, with cells between 2  $\mu\text{m}$  and 4  $\mu\text{m}$  in diameter (Figure 1D). Some isolates presented this morphology, while others consisted of small Gram-negative bacilli but with a morphology very similar to that displayed by *Azospirillum*, *Beijerinckia*, *Herbaspirillum* and *Derxia* (Figure 1E). However, it is known that *Azotobacter* is a pleiomorphic microorganism (4). In general, morphological identification and characterization are not necessarily useful in defining the genus as it was the case of isolates C3BR and C5BR, which exhibited morphological characteristics similar to those reported for *Azotobacter* besides growing in nitrogen-free culture media but the molecular identification revealed that they do not belong to that genus. It has been reported that *Azotobacter* has the capacity to produce soluble pigments (1, 4), this can be a useful tool in the characterization of some *Azotobacter* species. Brown or black pigmentation in the Ashby-Benzoate agar allowed in this study the differentiation between *A. chroococcum* and *A. nigricans* (22). Isolates C1CO, C5E, C1T, C3T and C5T exhibited morphological traits and similar pigmentation to those displayed by *A. chroococcum* and *A. nigricans* (Figure 1H and Table 1). On the another side, isolates C3CA and C4E presented colonies with brown-yellow pigmentation, Gram-negative bacilli, and catalase and oxidase negative tests, suggesting that they can be aerobic FNFB that do not belong to the genus *Azotobacter*. In the biochemical identification, tests allowing the differentiation between genera and species were used. An example is that *A. vinelandii* is the only species capable of assimilating ramnose and using it as carbon source. In addition, all *Azotobacter* species have the capacity to produce oxidases and catalases for the protection of their nitrogenase. Universal primers (Y1-Y3) have been used in FNFB 16S rDNA amplification (2, 17, 20) although not specific for the genus *Azotobacter*. FNFB characterization can be done based on several different genes coding for many functions, as is the case of the gene *nif* that codifies the

nitrogenase enzyme which drives the atmospheric nitrogen fixation (21, 26), in this work a partial sequence of *nifH* gene was amplified in several isolates including in the tree groups of ARDRA (data not shown). Nevertheless, both the 16S rDNA characteristics and its vertical transmission render this gene as an excellent molecular marker (23, 39). ARDRA is a molecular technique that allows establishing phylogenetic relationships between individuals. The enzymes used must generate high levels of polymorphism for specific groups to facilitate the detection of intra or inter-specific significant differences (14). The comparison between ARDRA and *in silico* analysis revealed that ARDRA is highly useful in establishing phylogenetic relationships between isolates and in clarifying the taxonomy. In general, the percentages of polymorphism of enzymes used in this work were relatively high, 16.5% on average, and similar to those reported by Aquilanti *et al.* (2) who obtained polymorphisms of 22%, 23% and 16% for *AluI*, *HpaII* and *RsaI*, respectively. A directly proportional relationship was demonstrated between the percentages of polymorphism and the ribotypes produced. UPGMA showed three main groups of similarity (Figure 3). We noted that group I isolates are found only in crop-grown soils located in Sogamoso, whereas group II and III isolates occur in crop-grown soils in both localities. Based solely on soil characteristics and the grown crops, clustering may differ from that one obtained with ARDRA. On the other hand, there was no relationship between the groups and the pH of crop-grown soils, although most isolates of group II were recovered in pH with values between 7.06 and 7.44. C5T and C1Z always showed irregular patterns for each enzyme and isolate C5E displayed a unique band with *RsaI* (Figure 2); these unique bands are particularly important since they can be useful for designing specific primers. When comparing the patterns generated in ARDRA with those obtained from the *in silico* restriction, a great consistency between number of bands and their corresponding molecular weights was observed in some cases. In other cases, this consistency results only for one or

two enzymes. Inconsistencies could be due to the fact that the lengths of some of the GenBank 16S rDNA sequences do not fully match with the lengths of the sequences of our isolates, probably because the primers used differed from ours. By this reason the molecular weights of the bands could differ by a few base pairs, especially in fragments of the flanking regions of the gene, considering as similar those bands that differed by 15-25 bp. On the other hand, in some isolates the presence of bands of greater molecular weights was observed after the digestion, suggesting that some copies of the gene were not completely digested. Isolates of group I of the ARDRA (Figure 3) showed restriction patterns similar to those obtained from the sequences of *Azotobacter* species used in the *in silico* restriction, whereas the majority of isolates of group II did not show this similarity. With respect to isolates of group III, a special finding was isolate C5T that produced patterns identical to *A. chroococcum* (accession number AY353708). In general, the BLASTn analysis carried out with partial sequences of the 16S rDNA gene obtained from every isolate revealed for group I similarity values between 74% and 84% with sequences of *A. vinelandii*; isolate C5CA showed a similarity of 78% (EF620439). Strain C5CO showed a similarity of 91% with *A. nigricans*, confirming its preliminary identification. Regarding isolates of group III from ARDRA, isolate C5T showed a similarity of 99% with *A. chroococcum* (AY353708). In conclusion, from the 15 isolates obtained from vegetable-grown fields, 14 exhibited phenotypical similarity with species of the genus *Azotobacter* and 13 showed restriction patterns similar to the ones obtained with the sequences of *Azotobacter* in the *in silico* restriction. Isolate C5T obtained from a tomato-grown field (*Lycopersicon esculentum*) could be identified as *A. chroococcum*. It is important to note that the short length of the sequences analyzed with BLASTn did not suffice to obtain a clarifying view of the taxonomic position of the majority of isolates. However, ARDRA proved to be a molecular tool that allows the identification of interspecific ribotypes of FNFB using a few polymorphic enzymes. The analysis of diversity is

an important factor in the measurement of quality of agricultural soils. On the other hand, it is necessary to design species-specific primers for the genus *Azotobacter* because of its large importance as atmospheric nitrogen fixing and plant growth promoting rhizobacteria.

#### ACKNOWLEDGMENT

This research was supported by the International Federation of Catholic Universities (CIC-FIUC). We thank the Environmental Biotechnology Unit and the Plant Biotechnology Unit of the Pontificia Universidad Javeriana for their collaboration.

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