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Diazotrophic bacteria associated with banana (*Musa* spp.).

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Abstract

Nitrogen-fixing bacteria were isolated from surface sterilized banana (*Musa* spp.) plants and constituted a minor proportion of banana endophytic bacteria. Some isolates were characterized by alloenzyme profiles, biochemical tests, 16S rRNA and *rpoB* partial gene sequences, plasmid profiles and plant colonization. A large group of enterobacterial isolates that could not be clearly affiliated, most of them ascribed to group I (with characteristics of *Enterobacter cloacae*) were the diazotrophs most frequently found in banana. Different *Klebsiella* spp. and *Rhizobium* sp. were identified as well. *Klebsiella* spp. were isolated from inside the roots and stems of plants grown in the two geographical regions sampled and from tissue culture-derived plantlets. *Rhizobium* sp. isolates were obtained only from Colima where bananas are grown extensively. Group I isolates and *Rhizobium* sp. could be re-isolated from surface-sterilized banana derived from tissue culture at five months after inoculation and significant increases in stem and leave fresh weight were obtained with some of the isolates.

Introduction

The isolation and characterization of diazotrophic endophytic bacteria from surface sterilized plants is a growing field of research interest and a large phylogenetic diversity of bacteria associated with agronomically important crops such as rice, sugar cane and maize has been found. A rationale for the study of endophytic diazotrophs is to extend biological nitrogen fixation to important non-legume crops, thereby displacing the use of chemical N fertilizers. The inside of plants constitute adequate habitats for bacterial colonization and novel bacterial groups may be encountered therein. Nevertheless, although beneficial effects of associated diazotrophic bacteria on their hosts are known, only in a few cases is the growth promotion due to nitrogen fixation (James, 2000). Plant growth may be stimulated by bacterially-produced phytohormones (Okon and Labandera-González, 1994; Tien et al., 1979; Yanni et al., 2001), vitamin-related products (Phillips et al., 1999) or by pathogen supression activities (Bashan and de Bashan, 2002; Haque and Ghaffar, 1993).

A current problem in the analysis of the effects of the inoculated endophytes on plants is the growth promotion mediated by the resident bacteria that masks the effects of the applied inocula. To eliminate endophytic bacteria, plants derived from tissue culture with or without antibiotic treatments, are sometimes used (Holland and Polacco, 1994; Leifert et al., 1991).

Bananas are propagated vegetatively and tissue culture is a common source of plants for agricultural fields in many places (Robles-González and Orozco-Romero, 1996). Agricultural practices in commercial crops of banana include chemical nitrogen fertilization to increase yield using over 200 kg N ha^{-1} in many countries including Brazil (Weber et al., 1999). Banana is the most widely consumed fruit in the world and in some countries it is the main source of nutrients. Commercial bananas have a narrow genetic diversity (Powledge, 1996). When this work started there was no information on banana associated nitrogen-fixing bacteria, since then some banana bacterial diazotrophs

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have been reported. By morphological and physiological characterization, bacteria similar to *Herbaspirillum* were identified from non-sterilized banana tissues that were different from species of *Herbaspirillum* isolated from gramineous plants; other banana diazotrophs not corresponding to *Herbaspirillum* were also encountered but their taxonomic affiliation was not defined (Weber et al., 2001). Species specific oligonucleotides directed to 16S rRNA genes were used by the same authors to identify diazotrophs from water-washed banana tissue samples as *Azospirillum brasilense* and as *Herbaspirillum*-like bacteria (Weber et al., 1999). Subsequently they reported the patterns and sequences of 16S rRNA genes that allowed the identification of banana isolates as *Herbaspirillum*, *Burkholderia* and *Ochrobactrum*-related bacteria (Cruz et al., 2001). Micropropagated banana plants grew faster upon inoculation of *Herbaspirillum*-like and *Burkholderia cepacia* related bacteria (Weber et al., 2000).

The aim of this work was to isolate and characterize putative endophytic nitrogen-fixing bacteria from surface sterilized banana plants grown in fields in Mexico, and resident endophytes in small plants derived from tissue culture.

Materials and methods

Plant surface sterilization

Bacteria were isolated from fields of two regions separated by 800 km: Colima (104 °W, 19 ° N) is home to many important banana plantations while Morelos (99 °W, 18.5 ° N) has small traditional farms where bananas are grown only sporadically. The amount of fertilizer normally used in Colima is 200 kg N ha^{-1}, 75 kg P ha^{-1} and 150 kg K ha^{-1}. No fertilizer is used in Morelos. Different banana genotypes such as Enano Gigante (*Musa* spp. Sub-Group *cavendish* AAA), hybrid cultivars FHIA 01, FHIA 21(AAAB), and Dominico (Sub-Group plantain AAB) were sampled. Enano Gigante is the banana commercial variety most extensively grown. Plant roots, stems or leaves were thoroughly washed with water, rinsed briefly with 96% ethanol and washed again with sterilized distilled water. Tissue culture samples were submerged in 2.4% sodium hypochlorite for 2 min and field borne samples for 10 min. Surface sterilization tests were performed by placing the samples on plates with the different media described below.

Bacteria isolation, bacterial reference strains and transposon tagging

Plant samples were macerated in a mortar in sterile water in a 1:10 (weight/volume) ratio.

The solid and semisolid media used were acetic LGI (Cavalcante and Döbereiner, 1988), acetic LGI modified with lower sugar (10 g L^{-1}sucrose), acetic LGI (10 g sucrose L^{-1}) with plant banana extract (2 g of macerated shoots in 10 mL water centrifuged and filter sterilized, referred here as plant extract medium), NFb (Döbereiner et al., 1976) and the medium used for *Azotobacter* and *Azomonas* strains in Reinhold-Hurek et al. (1993) designated here as Azo medium. Other solid media used for isolation and purification were SSM medium (Reinhold et al., 1986), Luria broth (LB), PY (3 g yeast extract, 5 g peptone and 0.7 g calcium chloride L^{-1}), MacConkey, Congo Red (Rodríguez Cáceres, 1982). Solid media used for plant isolation with agar (14 g L^{-1}) in plates were supplemented with yeast extract 0.05 g L^{-1} (except Azo) and cycloheximide (100 mg L^{-1}) Extensive colony purification was performed to achieve single strain cultures.

The following additional *Klebsiella* isolates from different hosts were used in comparison to banana *Klebsiella* isolates: from sugar cane T29A, T3A, T40B, T42A, T44C; from maize 3 (Caballero-Mellado et al., unpublished). The reference strains used were *Gluconacetobacter diazotrophicus* PAl 5^T, *Rhizobium etli* CFN42, *Rhizobium undicola* LMG11875, *Sinorhizobium* sp. CFNEA156, *Klebsiella pneumoniae* ATCC 13883^T, *Klebsiella planticola* ATCC 33531^T, *Klebsiella oxytoca* ATCC 13182^T and *Klebsiella terrigena* ATCC 33257^T.

A culture of the *Rhizobium* banana isolate F5R19 was crossed mated with *E. coli* S17-1λpir carrying pFAJ1819 (Xi et al., 2001) that contains a Tn*5-gusA* transposon that confers resistance to kanamycin. Transposon-tagged colonies of F5R19 were selected in kanamycin (Km, 60 mg L^{-1}) and tested for growth in minimal medium (González-Pasayo and Martínez-Romero, 2000) to discard auxotrophs. Five independent colonies were chosen to be used in banana plant colonization assays.

API 20E galleries

Two colonies of enterobacteria isolates and type strains were resuspended in 5 mL of sterilized distilled water and used to inoculate test plates from API 20E identification system (bioMérieux sa, Marcy l'Etoile,

France). Plates were incubated at 37 °C for 24 or 48 h and activities were revealed according to manufacturers instructions and recorded using APILAB plus automated interpretation.

Acetylene reduction activity (ARA assays)

Bacteria were grown for 3 days in different semisolid media (Table 1) without nitrogen containing 2.3 g of agar L^{-1} in 10 mL vials filled with 5 mL of media. Acetylene was added to attain a concentration of 12% (v/v) and ethylene production was determined after 12 h in a Varian 3300 gas chromatograph as described (Rogel et al., 2001).

Multilocus enzyme electrophoresis (MLEE)

The procedures described by Selander et al. (1986) were used with bacterial extracts derived from liquid cultures in different liquid media. The following metabolic enzymes were assayed: alcohol, glucose-6-phosphate, glutamate, isocitrate, leucine, lysine, malate and xanthine dehydrogenases, phosphoglucose isomerase and phosphoglucomutase.

Primers for PCR reaction and sequence analysis

Primers rD1 and fD1 (Weisburg et al., 1991) were used to amplify almost complete copies of 16S rRNA genes, also used were 16S rRNA gene internal primers for enterobacteria, KC856R (GCTCCGGAAGCCACGCCTCAA) and 533F (Dojka et al., 1998). Primers CM_7 (AACCAGTTCCGCGTTGGCCTGG) and CM_{31b} (CCTGAACAACACGCTCGGA) (Mollet et al., 1997) were for the amplification of *rpoB* genes. PCR products were sequenced as described (Rogel et al., 2001). Neighbor-joining was used to construct the dendrograms using Jukes-Cantor distances. *nifH* genes were amplified with polF and polR primers (Poly et al., 2001).

Plasmid profiles

Modified Eckhardt gels of SDS-agarose (Hynes and McGregor, 1990) were used to visualize plasmids from banana isolates and reference strains. The *Klebsiella* plasmid patterns were blotted and hybridized to the *nifH* gene of *Rhizobium etli* CFN42 (Wang et al., 1999) as described (Martínez-Romero et al., 1991).

DNA hybridization

Total DNA was extracted with the Genomic Prep Cells and Tissue DNA Isolation kit (Amersham, Piscataway, NJ, USA) or with the Quantum Prep, Aqua Pure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA, USA). Nylon filters with blotted restricted DNA from a diversity of strains in respective lanes were hybridized to total DNA labelled with ^{32}P using Rediprime (Amersham). Probes for hybridization were DNAs of *K. pneumoniae* ATCC13883 or 6A2 in the enterobacteria hybridization assays and DNA of F5R19 in the *Rhizobium* assays. Total hybridization was calculated as the percent of the hybridization of the homologous strain.

Banana colonization assays

One month old plantlets derived from tissue cultures of Enano Gigante that were maintained in closed glass flasks at 28 ° C in a growth chamber were inoculated with 10^6 bacteria and transferred to pots with vermiculite and Fahraeus N-free medium (Fahraeus, 1957). Plants were watered with Fahraeus N-free medium as required. After one or 5 months plantlets were surface sterilized as described above and macerated. Macerates were diluted and plated on the media described. Three plants per strain were analyzed for the presence of endophytic bacteria at each sampling time. Km^r colonies were counted to quantify F5R19 banana colonizing bacteria. Leaf and root fresh biomass were determined at 5 months after inoculation.

Plant nodulation assays

Acacia farnesiana seeds were treated with concentrated sulfuric acid for 20 min and then washed extensively with sterile water, seeds were germinated in agar-H_2O plates for 3–5 days, transferred to sterile vermiculite with Fahraeus N-free medium (Fahraeus, 1957) and inoculated with 100 μL of bacterial suspensions of *R. etli* strain CFN42, *Rhizobium* sp. CFNEI156 (Toledo et al. 2003) or isolated banana endophytes E2C4, E2C6, A3R3, ESR13a, FSH16b, F5R19. Nodule occupancy was verified by the isolation of bacteria following described procedures (Martínez-Romero and Rosenblueth, 1990).

Phenotypic analysis

Resistance to antibiotics, different substrate utilization and enzymatic reactions were performed using the

Table 1. Nitrogen fixing banana isolates

Strain	Geographic origin	Plant organ	Medium of isolation	ARA[a] nmolC_2H_4/h/vial	ARA Semisolid Medium tested
Group I *Enterobacter* sp.					
A4T2	Colima	Stem	Congo red	9.86	NFb
B5R5	Colima	Root	Plant extract	27.36	Plant extract
C1T5	Colima	Stem	Acetic LGI	121.87	Acetic LGI
A1T1	Colima	Stem	Acetic LGI	33.29	Acetic LGI
C1C2	Colima	Root	Acetic LGI	109.56	Acetic LGI
C1C3	Colima	Root	Acetic LGI	77.01	Acetic LGI
C1T4	Colima	Stem	Acetic LGI	47.40	Acetic LGI
C2R2	Colima	Root	LGI (10 g sucrose)	31.43	LGI(10 g sucrose)
C2R3	Colima	Root	LGI (10 g sucrose)	40.26	LGI(10 g sucrose)
E2C7	Colima	Root	LGI (10 g sucrose)	35.31	LGI(10 g sucrose)
A3C1	Colima	Root	Azo	56.43	Azo
A3C2	Colima	Root	Azo	87.61	Azo
A3R3	Colima	Root	Azo	64.11	Azo
B3C4	Colima	Root	Azo	120.53	Azo
B3T5	Colima	Stem	Azo	81.77	Azo
B3T6	Colima	Stem	Azo	83.64	Azo
C3C7	Colima	Root	Azo	132.31	Azo
C3C8	Colima	Root	Azo	168.64	Azo
C3C9	Colima	Root	Azo	268.01	Azo
C3T11	Colima	Stem	Azo	125.48	Azo
D3R12	Colima	Root	Azo	117.74	Azo
E3C13	Colima	Root	Azo	94.10	Azo
E3H14	Colima	Leaves	Azo	63.34	Azo
E3R15	Colima	Root	Azo	117.68	Azo
E3T17	Colima	Stem	Azo	44.14	Azo
F3R22	Colima	Root	Azo	81.34	Azo
F3R23	Colima	Root	Azo	78.16	Azo
C4T4	Colima	Stem	Congo red	61.76	NFb
E4H6	Colima	Leaves	Congo red	76.41	NFb
E4H7	Colima	Leaves	Congo red	49.78	NFb
A5T1	Colima	Stem	Plant extract	66.80	Plant extract
C5C8	Colima	Root	Plant extract	48.04	Plant extract
C5T9	Colima	Stem	Plant extract	31.66	Plant extract
E5T14	Colima	Stem	Plant extract	37.22	Plant extract
A3C1a	Colima	Root	Azo	125.67	Azo
Group II *Citrobacter* sp.					
III	Morelos	Leaves	Plant extract	67.00	Plant extract
IIIA	Morelos	Leaves	Plant extract	59.31	Plant extract
Group III *Klebsiella* sp.					
6A2	Colima TC[b]	Leaves	Acetic LGI	95.04	Acetic LGI
VI	Morelos	Stem	LGI (10 g sucrose)	147.31	LGI(10 g sucrose)
2S9	Colima TC[b]	Root	Acetic LGI	75.57	Acetic LGI
2S12	Colima TC[b]	Root	Acetic LGI	63.92	Acetic LGI
4A3	Colima TC[b]	Leaves	Acetic LGI	59.42	Acetic LGI
6A1	Colima TC[b]	Leaves	Acetic LGI	83.21	Acetic LGI

Table 1. Continued.

Strain	Geographic origin	Plant organ	Medium of isolation	ARA[a] nmolC_2H_4/h/vial	ARA Semisolid Medium tested
6A6	Colima TC[b]	Leaves	Acetic LGI	34.11	Acetic LGI
3S6	Colima TC[b]	Root	LGI (10 g sucrose)	66.29	LGI(10 g sucrose)
4A5	Colima TC[b]	Leaves	LGI (10 g sucrose)	35.06	LGI(10 g sucrose)
5S1a	Colima TC[b]	Root	LGI (10 g sucrose)	67.21	LGI(10 g sucrose)
5A1	Colima TC[b]	Leaves	LGI (10 g sucrose)	34.10	LGI(10 g sucrose)
3S3	Colima TC[b]	Root	Azo	42.00	Azo
3S4	Colima TC[b]	Root	Azo	67.31	Azo
3S5	Colima TC[b]	Root	Azo	66.29	Azo
5A1a	Colima TC[b]	Leaves	Azo	33.24	Azo
Group IV *Klebsiella sp.*					
F2R9	Colima	Root	LGI (10 G sucrose)	12.00	LGI(10 g sucrose)
C1Tb	Colima	Stem	Acetic LGI	43.07	Acetic LGI
D1T8	Colima	Stem	Acetic LGI	110.97	Acetic LGI
VIA	Morelos	Stem	LGI (10 g sucrose)	120.05	LGI(10 g sucrose)
VIB	Morelos	Stem	LGI (10 g sucrose)	131.10	LGI(10 g sucrose)
Group V *Rhizobium* sp.					
F5R19	Colima	Root	Plant extract	–	Plant extract
F5H16b	Colima	Leaves	Plant extract	–	Plant extract
A3R3	Colima	Root	Azo	–	Azo

[a] ARA: Acetylene reduction assay, measured for bacterial cultures grown in the indicated media.
[b] TC: Plantlets derived from tissue culture.

MICRONAUT-E system (Merlin-Diagnostika Gmbh, Bornheim-Hersel, Germany).

Results

Isolation and identification of bacteria

The isolation of bacteria from surface sterilized stem, leaves, and roots normally allows the recovery of putative endophytic bacteria. Growth in semisolid nitrogen free medium has been a useful strategy to select for nitrogen fixing bacteria that are subsequently tested for acetylene reduction activity. All isolates, obtained from banana, selected by these combined criteria were grouped by morphological similarities and phenotypic characteristics. Alloenzyme patterns, PCR-RFLP and sequence of 16S rRNA genes, plasmids profiles and *rpoB* gene sequences were determined for some representatives of each group. *rpoB* gene sequences have been useful in enterobacteria systematics (Mollet et al., 1997), *rpoB* codes for the β-subunit of the RNA polymerase. DNA-DNA hybridization was performed to further define the relationships of the type strains of the groups that were found to be closely related to the banana isolates.

Out of 1453 isolates from surface sterilized banana tissues, only 57 isolates reduced acetylene and were retained (Table 1). Despite some isolates only producing low levels of ethylene (e.g. B5R5, A4T2 and F2R9), they were considered nitrogen-fixing bacteria because they also have *nifH* genes detectable by PCR using primers polF and polR and the ARA detected was reproducible. Negative controls and all of the non-nitrogen-fixing isolates gave undetectable ethylene in the 12 h assays used. With isolates obtained from some of the media as SSM or LB we did not detect acetylene reduction activity. Nitrogen-fixing isolates were divided into five morphological groups (Table 1). Four of these groups encompassed bacteria with colonies appearing after one day of incubation on plates with different media and having duplication times of about 35 minutes in liquid LB medium, a rich medium that allows fast generation times with Enterobacteria. Some of these bacteria were further analyzed and found to correspond to Enterobacteria from the analysis of 16S gene sequences and from the results of API 20E galleries. Their diversity and group-

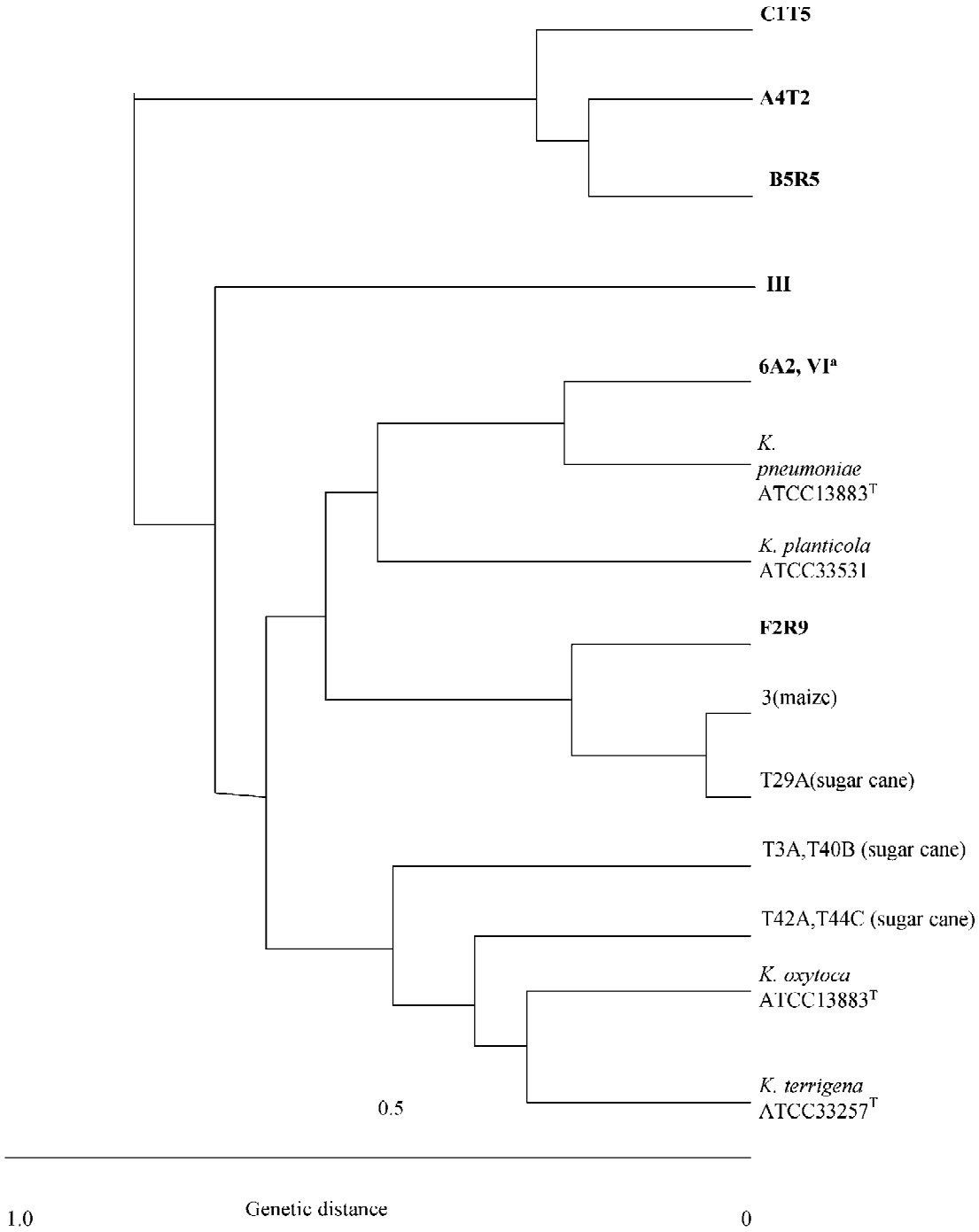

Figure 1. Genetic relatedness of *Klebsiella* isolates from banana, sugar cane and maize plants and group I isolates defined by MLEE analyses. The dendrogram was constructed from data for 10 metabolic enzymes. Banana isolates are bold. [a] 18 more *Klebsiella* isolates correspond to the same ET.

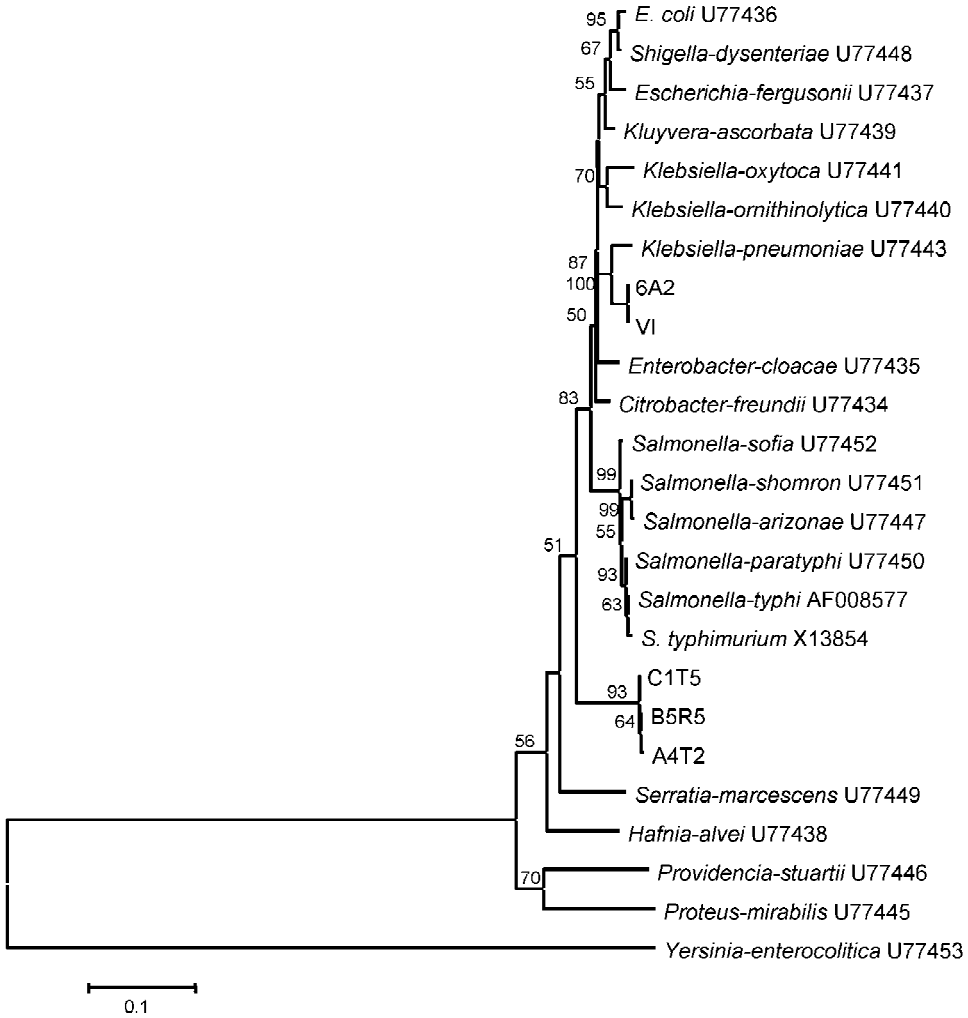

Figure 2. Relatedness among enterobacteria banana isolates and reference strains based on *rpoB* gene sequences.

ings were established from their metabolic enzyme patterns using 10 enzymatic activities (Figure 1).

Group I

The most frequent nitrogen-fixing bacteria with 35 isolates, representing almost 60% of the total nitrogen fixing isolates were obtained from surface sterilized banana stems, leaves and roots from Colima (Table 1). They formed a group of related bacteria placed at a genetic distance of 0.85 from other clusters of enterobacteria banana isolates based on alloenzyme analysis (Figure 1). Strains CIT5, B5R5 and A4T2 were found to be similar to *Enterobacter cloacae* (accession number AF047175) with 99% identity in the sequence of 16S rRNA genes analyzed but from the sequence of *rpoB* genes group I isolates (CIT5, B5R5 and A4T2) constituted an independent cluster (Figure 2).

API 20E galleries analysis placed isolate A4T2 in *Enterobacter cloacae* with a probability of 96.1% while isolates B5R5 and CIT5 had lower probability values (75.8%). A4T2, B5R5 and CIT5 produced acid in medium with glucose and were motile (like *E. cloacae* rice isolates, Ladha et al., 1983). Isolates B5R5 and CIT5 were identified as *E. clocae* by the MICRONAUT E-system.

Group II

A minority group of isolates constituting 2% of the nitrogen-fixing endophytes were obtained from leaves of Morelos plants. From the 16S rRNA gene sequence

similarity, strain III was only related to *Citrobacter* (accession number AF0253669) albeit not closely (96%). Isolates belonging to this group formed pink colonies in Acidic Koser Citrate medium. They were non motile and grew at 37 °C and they were assigned to *Pantoea agglomerans* by the MICRONAUT-E system.

Groups III and IV: Klebsiella *spp.*

Klebsiella isolates were obtained from inside stems and leaves from the field samples from both geographical regions sampled and also from inside roots from tissue culture (Table 1). Two groups (groups III and IV) were distinguished among *Klebsiella* banana isolates by morphology but also by MLEE. *Klebsiella* spp. isolates obtained previously in Mexico from sugar cane (T29A, T3A, T40B, T42A and T44C) or maize (strain 3) were included for comparison. All *Klebsiella* isolates from banana, sugar cane or maize and the reference strains grew at 37 °C on MacConkey solid media.

Using API 20E galleries the isolate 6A2 was identified as *K. pneumoniae* at 90.4% identity and *Klebsiella* isolates 6A2 and VI were found to correspond with very good agreement to *Klebsiella pneumoniae* subsp. pneumoniae using the MICRONAUT-E system, but they differed by their inability to use rhamnose and adonitol. In multilocus enzyme electrophoresis assays banana isolates 6A2, VI and 13 others were closely related to *K. pneumoniae* at a genetic distance of 0.3 using 10 metabolic enzymes while other *Klebsiella* isolates from banana (such as F2R9), maize, and sugarcane were at a genetic distance of 0.63 from *K. pneumoniae.* Some sugar cane isolates were found to be related to the type strains of *K. oxytoca* and *K. terrigena* (Figure 1).

Banana *Klebsiella* isolates 6A2, VI, F2R9 as well as type strains from *K. pneumoniae*, *K. oxytoca, K. terrigena* and *K. planticola* grew in Acidic Koser Citrate Medium (Bruce et al., 1981), which is used to identify *Klebsiella* (Grimont et al., 1992), forming yellow mucoid colonies while group I isolates CIT5, A4T2 and B5R5 formed pink colonies. Banana *Klebsiella* isolates were non motile like *K. pneumoniae* and *Klebsiella* rice isolates (Ladha et al., 1983).

16S rRNA gene sequences were obtained from *Klebsiella* spp. 6A2 (from tissue culture), VI from Morelos and F2R9 from Colima. Sequence identities of 97, 97 and 96% were found when 6A2, F2R9 and VI, respectively, were compared to *K. pneumoniae* accession numbers AB074192, AF009170, AF228920. The analysis of the *rpoB* gene sequence from 6A2 and VI showed that they were clearly related to *Klebsiella* (Fig. 2). The results of total DNA hybridization showed that *Klebsiella* sp. 6A2 was related to two *Klebsiella* isolates, one from maize (strain 3) and one from sugar cane (T29A) with hybridization values of 40 and 39%, respectively, and then to *K. pneumoniae* ATCC13883^T with hybridization of 30%. DNA hybridization of 6A2 to the total DNAs of *K. oxytoca*, *K. planticola* and *K. terrigena* was 7–10%, and to strain III from group II was 5%. Using *K. pneumoniae* ATCC 13883 as a probe, hybridization values of 20–23% were obtained with banana *Klebsiella* isolates 6A2, V1, F2R9 and 16% with *K. planticola*.

Group V: Rhizobium *sp.*

The fifth morphological group of nitrogen fixing banana bacteria was isolated from inside leaves and roots of Dominico and FHIA21 plants from Colima. These isolates formed colonies at 3 days. One of them, F5R19, resembled *Rhizobium undicola* (accession number AUY17047) on the basis of the 16S rRNA gene sequence (with 98% identity). Based on this result we determined alloenzyme patterns of some of these isolates compared with *Rhizobium undicola* type strain LMG11875 and found that isolates F5R19, F5H16b and A3R3 were related by their metabolic enzyme patterns but also by PCR-RFLP patterns of 16S rRNA genes to the *Rhizobium undicola* strain tested (Figure 3). In total DNA–DNA hybridization assays, when DNA of F5R19 was used as a probe, hybridization was 20% with DNA of *R. undicola* LMG11875, 60% with A3R3 and 100% with F5H16b. All *Rhizobium* strains tested (*R. etli* CFN42, *R. undicola* LMG11875) and rhizobia banana isolates F5R19 and F5H16 did not grow in Acidic Koser Citrate medium. Banana isolates F5R19, F5H16b and A3C1a did not nodulate bean (*Phaseolus vulgaris*) but formed small, ineffective nodules on *Acacia farnesiana*. Average nodule number per *A. farnesiana* plants was 7 and all six plants tested were nodulated with F5R19. To confirm that the bacteria recovered from the *Acacia* nodules corresponded to the inoculated strain, plasmid patterns were compared, the bacteria re-isolated from different nodules of F5R19 in *Acacia* had plasmid profiles (plasmids of around 100, 500 and >1000 kb) identical to the original strain used as inocula (Figure 4). This plasmid profile is different from that observed with type strain *R. undicola* LMG11875 (de

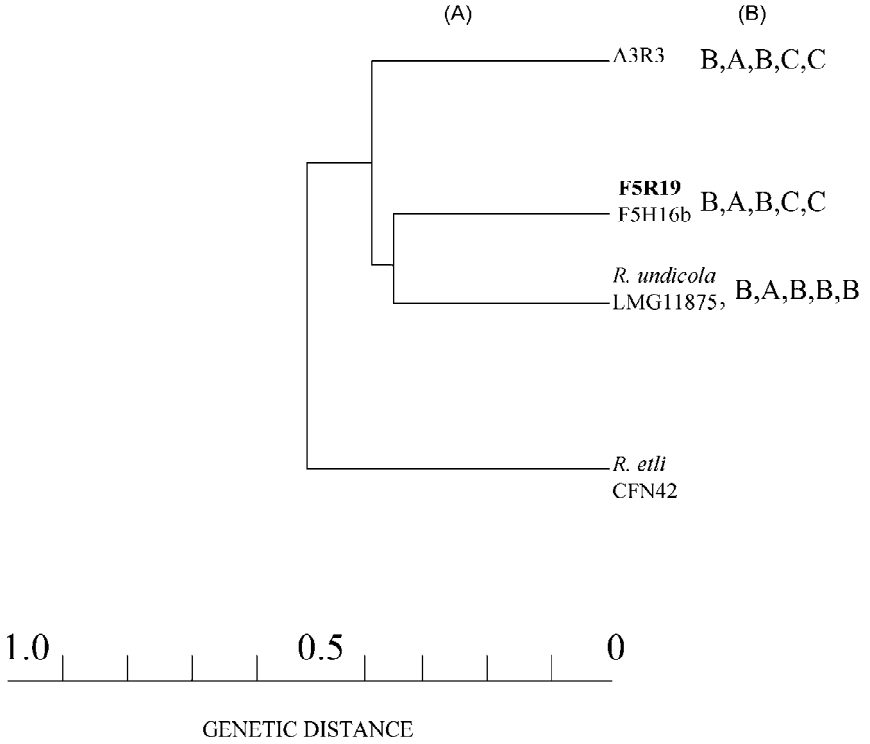

Figure 3. (A) Genetic relatedness of *Rhizobium* isolates from banana plants defined by MLEE analysis. The dendrogram was constructed from data for 10 metabolic enzymes. (B) 16S rRNA digestion patterns of PCR products of almost full-length genes. Each letter designates the digestion pattern obtained with the following restriction enzymes: *Dde*1, *Hinf*1, *Msp*1, *Sau*3A1, *Rsa*1. Banana isolates are bold.

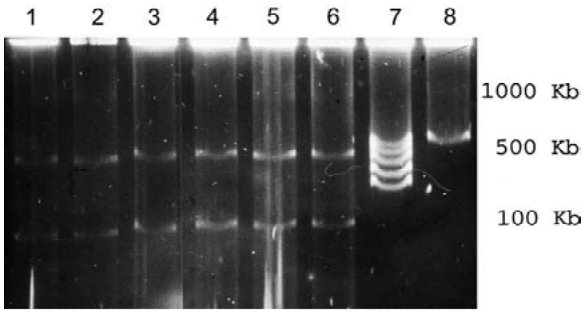

Figure 4. Plasmids visualized by a modified Eckhardt procedure. Lane (1–5) bacteria isolated from *Acacia* nodules of plants inoculated with F5R19; (6) banana *Rhizobium* isolate F5R19; (7) *R. etli* CFN42; (8) *R. undicola* LMG 11875^T.

Lajudie et al., 1998) and from A3R3, which carries plasmids of around 100, 400 and 1000 kb.

Table 2. Population of endophytic bacteria reisolated from surface sterilized banana plants previously inoculated with the indicated strain

Strain	(Cfu g [fresh wt]$^{-1}$)[a] Parts of the plant	
	Shoot	Root
Enterobacter-like (group I)		
A4T2	1.00×10^4	1.24×10^5
C1T5	1.06×10^5	2.38×10^5
B5R5	0.00	7.80×10^4
A3C1a	5.60×10^5	2.24×10^5
Rhizobium sp. (group V)		
A3R3	7.15×10^4	3.18×10^5
F5H16b	5.00×10^4	5.00×10^5
F5R19	2.00×10^3	4.70×10^5

[a] Results are means of three plants, the analysis was made 5 months after inoculation.

Re-isolation assays

Plantlets derived from tissue culture were inoculated with *Klebsiella* isolates 6A2 and VI, isolate III (from group II), group I isolates A4T2, C1T5, B5R5, and A3C1a and *Rhizobium* isolates F5R19, F5H16b, and A3R3. Reisolated bacteria were identified by their morphology on plates of PY and MacConkey media and by their resistance to kanamycin in the case of the *Rhizobium* isolate F5R19. No kanamycin resist-

ant colonies were obtained from non- inoculated plant extracts. In all cases the inoculated endophyte was recovered from the inoculated plants. CFU recovered from surface sterilized bananas ranged from 10^4–10^5 bacteria per g of fresh tissue for group I bacteria and 10^3–10^5 bacteria per g of fresh tissue for *Rhizobium* inoculated plants (Table 2). Non-inoculated plants did not have any *Rhizobium* strains nor strains morphologically similar to group I or group II but had inside stems and roots other putative endophytic bacteria that were not analyzed (at around 10^5 CFU per gram) and *Klebsiella*-like isolates similar to isolate 6A2.

Inoculation did not cause any detrimental effect on the growth of plants, on the contrary, stimulation of plant development was recorded in some cases, specially with group I bacteria and with *Rhizobium* F5R19. At 5 months after inoculation of tissue culture-derived plantlets, significant increases of plant fresh weight (stems and leaves) were obtained ($p<0.05$, t 'student' test) with group I isolate B5R5 and *Rhizobium* strain F5R19. Increases of over 60% were recorded in both cases.

Discussion

In rice diazotrophs constitute a minor fraction of all associated bacteria (Barraquio et al., 1997) and the possibility of promoting nitrogen fixation in rice by increasing the proportion of diazotrophs has been suggested (Ladha and Reddy, 2000). With the experimental procedures we used, only a low proportion of diazotrophs was obtained from banana.

Based on biochemical characteristics, nitrogen-fixing bacteria associated with leaf sheaths and roots of rice were identified as *Enterobacter cloacae* and *Klebsiella planticola* (Ladha et al., 1983 and references therein). *Enterobacter* strains have been isolated from stems of wild and cultivated rice (Elbeltagy et al., 2001) and from sugar cane (Mirza et al., 2001). *Pantoea* and synonymus *Enterobacter* strains have also been isolated as endophytes of cotton (Sturz et al., 1997) and alfalfa (Phillips et al., 1999). *E. cloacae* isolates when inoculated into plants promoted rice development (Fujii et al., 1987) and nitrogen fixation in rice (Elbeltagy et al., 2001; Mirza et al., 2001) and in sugar cane. Our preliminar results showed that different group I strains enhanced shoot growth in comparison to non inoculated control plants. Group I isolates were first considered to belong to *Enterobacter* genus but the distinctive grouping by *rpoB* gene sequences led us to leave their affiliation open. The dendrogram obtained from *rpoB* gene sequences is in good agreement to the published results (Mollet et al., 1997).

Historically, the first *Klebsiella* isolates were from clinical samples but they have also been frequently encountered in plants, water and soils (Grimont et al., 1992). *K. pneumoniae*, *K. oxytoca*, and *K. planticola* are all capable of fixing nitrogen. Recently a modification in the taxonomy of *Klebsiella* has been proposed and *K. planticola* and *K. terrigena* have been transferred to the genus *Raoultella* (Drancourt et al., 2001). The isolation of nitrogen-fixing *Klebsiella* from rice leaves (Ladha et al., 1983) and *K. pneumoniae* from maize (Palus et al., 1996) has been reported. The genomic differences among *Klebsiella pneumoniae* clinical and maize isolates were analyzed using microarrays (Dong et al., 2001). In maize, *Klebsiella pneumoniae* was located in the intracellular space of the stem cortex, and in the root epidermis near the cortex (Chelius and Triplett, 2000). The nitrogenase reductase of *K. pneumoniae* was detected with antibodies in maize plants amended with a carbon source (Chelius and Triplett, 2000) and these bacteria have been tested as maize inoculants in the US. (Triplett, pers. comm.).

Rhizobia have been safely used as plant inoculants in agriculture for more than a hundred years. *Rhizobium* spp. and *Azorhizobium caulinodans* have been found to colonize endophytically both legumes (O'Callaghan et al., 1999) and non-legumes (Engelhardt et al., 2000; Gough et al., 1997; Gutiérrez-Zamora and Martínez-Romero, 2001; O'Callaghan et al., 2000; Yanni et al., 1997) promoting plant growth in some cases. *Rhizobium undicola* was isolated from *Neptunia natans* in Senegal and described as a novel genus *Allorhizobium*, closely related to *Agrobacterium* and capable of effectively nodulating *Acacia* spp and *Faidherbia albida* (de Lajudie et al., 1998). Recently *Allorhizobium* has been emended to become *Rhizobium* (Young et al., 2001). The differences among F5R19 and *Rhizobium undicola* suggest that they may belong to different but related species. The isolation method that we used was not designed to select for *Rhizobium* and that may explain why we obtained very few *Rhizobium* isolates since *Rhizobium* strains do not normally fix nitrogen in free-living conditions not even in semigelified medium used for other diazotrophs. The fact that they were able to colonize banana tissues in good numbers and had plant growth promotion effects indicates that they are adapted to

these conditions. These isolates originally detected in semisolid medium for their capacity to reduce acetylene were subsequently found not to fix nitrogen in the same medium as expected for all rhizobia. In spite of the fact that *Rhizobium* sp. does not fix nitrogen in these conditions, our interpretation is that F5R, A3R3 and F5H16b have not lost the capacity to fix nitrogen; furthermore, a plasmid with *nifH* genes was found to be stably maintained (not shown). In contrast, in subsequent subcultures, group I and II isolates and *Klebsiella* isolates were found to reproducibly fix nitrogen in free living cultures even those giving ethylene production less than 50 nmol/hr/culture.

Rhizobia colonization to non-legumes seems to be promoted by a close physical association of legumes and non-legumes in some agricultural fields (Yanni et al., 1997). In this regard, native *Acacia* spp. trees are abundant in the banana crop area in Colima where banana endophytic rhizobia were encountered.

The endophytic populations that we isolated, namely γ-Proteobacteria and *Rhizobium,* are certainly different from the β-Proteobacteria (*Burkholderia* and *Herbaspirillum*) and *Ochrobactrum*-related bacteria obtained in Brazil as banana associated bacteria (Cruz et al., 2001; Weber et al., 1999). This may be in relation to the differences in media used and the bacterial isolation procedure but the effects of the different plant genotypes, the regional endophytic populations and the environmental conditions can not be discarded to account for the different results obtained.

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References

Barraquio W L, Revilla L and Ladha J K 1997 Isolation of endophytic diazotrophic bacteria from wetland rice. Plant Soil 194, 15–24.

Bashan Y and de-Bashan L E 2002 Protection of tomato seedlings against infection by *Pseudomonas syringae* pv. tomato by using the plant growth-promoting bacterium *Azospirillum brasilense.* Appl. Environ. Microbiol. 68, 2637–2643.

Bruce S K, Schick D G, Tanaka L, Jiménez E M and Montgomerie J Z 1981 Selective medium for isolation of *Klebsiella pneumoniae*. J. Clinical Microbiol. 13, 1114–1116.

Cavalcante V and Döbereiner J 1988 A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant Soil 108, 23–31.

Chelius M K and Triplett E W 2000. Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. Appl. Environ. Microbiol. 66, 783–787.

Cruz L M, Maltempi de Souza E, Weber O B, Baldani J I, Döbereiner J and Oliveira Pedrosa F 2001 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril). Appl. Environ. Microbiol. 67, 2375–2379.

Döbereiner J, Marriel I E and Nery M 1976 Ecological distribution of *Spirillum lipoferum* Beijerinck. Can. J. Microbiol. 22, 1464–1473.

de Lajudie P, Laurent-Fulele E, Willems A, Torck U, Coopman R, Collins M D, Kersteres K, Dreyfus B and Gillis M 1998 *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int. J. Syst. Bacteriol. 4, 1277–1290.

Dojka M A, Hugenholtz P, Haack S K and Pace N R 1998 Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64, 3869–3877.

Dong Y, Glasner J D, Blattner F R and Triplett E W 2001 Genomic interspecies microarray hybridization: rapid discovery of three thousand genes in the maize endophyte, *Klebsiella pneumoniae* 342, by microarray hybridization with *Escherichia coli* K-12 Open Reading Frames. Appl. Environ. Microbiol. 67,1911–1921.

Drancourt M, Bollet C, Carta A and Rousselier P 2001 Phylogenetic analyses of *Klebsiella* species delineate *Klebsiella* and *Raoultella* gen. nov., with description of *Raoultella ornithinolytica* comb. nov., *Raoultella terrigena* comb. nov. and *Raoultella planticola* comb. nov. Int. J. Syst. Evol. Microbiol. 51, 925–932.

Elbeltagy A, Nishioka K, Sato T, Suzuki H, Ye B, Hamada T, Isawa T, Mitsui H and Minamisawa K 2001 Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 67, 5285–5293.

Engelhard M, Hurek T and Reinhold-Hurek B 2000 Preferential occurrence of diazotrophic endophytes, *Azoarcus* spp., in wild rice species and land races of *Oryza sativa* in comparison with modern races. Environ. Microbiol. 2, 131–141.

Fahraeus G 1957 The infection of clover root hair by nodule bacteria studied by a single glass slide technique. J. Gen. Microbiol. 16, 374–381.

Fujii T, Huang Y -D, Higashitani A, Nishimura Y, Iyama S, Hirota Y, Yoneyama T and Dixon R A 1987 Effect of inoculation with *Klebsiella oxytoca* and *Enterobacter cloacae* on dinitrogen fixation by rice-bacteria associations. Plant Soil 103, 221–226.

González-Pasayo R and Martínez-Romero E 2000 Multiresistance genes of *Rhizobium etli* CFN42. Mol. Plant-Microbe Interact. 13, 572–577.

Gough C, Vasse J, Galera C, Webster G, Cocking E and Dénarié J 1997 Interactions between bacterial diazotrophs and non-legume dicots: *Arabidopsis thaliana* as a model plant. Plant Soil 194, 123–130.

Grimont F, Grimont P A D and Richard C 1992 The genus *Klebsiella. In* The Prokaryotes Second Edition. A Handboook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Eds. A Balows, H G Trüper, M Dworkin, W Harder and K -H Schleifer. pp. 2775–2796. Springer-Verlag, New York,

Gutiérrez-Zamora M L and Martínez-Romero E 2001 Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.). J. Biotechnol. 91, 117–126.

Haque S E and Ghaffar A 1993 Use of rhizobia in the control of root rot diseases of sunflower, okra, soybean and mungbean. J. Phytopathol. 138, 157–193.

Holland M A and Polacco J C 1994 PPFMs and other covert contaminants: is there more to plant physiology than just plant? Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 197–209.

Hynes M F and McGregor N F 1990 Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol. Microbiol. 4, 567–574.

James E K 2000 Nitrogen fixation in endophytic and associative symbiosis. Field Crops Res. 65, 197–209.

Ladha J K, Barraquio W L and Watanabe I 1983 Isolation and identification of nitrogen-fixing *Enterobacter clocae* and *Klebsiella planticola* associated with rice plants. Can. J. Microbiol. 29, 1301–1308.

Ladha J K and Reddy P M Eds 2000 The Quest for Nitrogen Fixation in Rice. Proceedings of the Third Working Group Meeting on Assessing Opportunities for Nitrogen Fixation in Rice, 9–12 Aug. 1999. Makati City (Philipines): International Rice Research Institute. Los Baños, Philippines. 359 pp.

Leifert C, Ritchie J Y and Waites W M 1991 Contaminants of plant-tissue and cell cultures. World J. Microbiol. Biotechnol. 7, 452–469.

Martínez-Romero E and Rosenblueth M 1990 Increased bean (*Phaseolus vulgaris* L.) nodulation competitiveness of genetically modified *Rhizobium* strains. Appl. Environ. Microbiol. 56, 2384–2388.

Martínez-Romero E, Segovia L, Mercante F M, Franco A A, Graham P and Pardo M A 1991 *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41, 417–426.

Mirza M S, Ahmad W, Latif F, Haurat J, Bally R, Normand P and Malik K A 2001 Isolation, partial characterization, and the effect of plant growth-promoting bacteria (PGPB) on micro-propagated sugarcane *in vitro*. Plant Soil 237, 47–54.

Mollet C, Drancourt M and Didier R 1997 *rpoB* sequence analysis as a novel basis for bacterial identification. Mol. Microbiol. 26, 1005–1011.

O'Callaghan K J, Davey M R and Cocking E C 1999 Xylem colonization of *Sesbania rostrata* by *Azorhizobium caulinodans* ORS571. *In* Highlights of Nitrogen Fixation Research. Eds E Martínez and G Hernández. pp. 145–147. Kluwer Academic/Plenum Publishers. New York.

O'Callaghan K J, Stone P J, Hu X, Griffiths D W, Davey M R and Cocking E C 2000 Effects of glucosinolates and flavonoids on colonization of the roots of*Brassica napus* by *Azorhizobium caulinodans* ORS571. Appl. Environ. Microbiol. 66, 2185–2191.

Okon Y and Labandera-González C A 1994 Agronomic aplicactions of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol. Biochem. 26, 1591–1601.

Palus J A, Borneman J, Ludden P W and Triplett E W 1996 A diazotrophic bacterial endophyte isolated from stems of *Zea mays* L., and *Zea luxurians* Itlis and Doebley. Plant Soil 186, 135–142.

Phillips D A, Joseph C M, Yang G P, Martinez-Romero E, Sanborn J R and Volpin H 1999 Identification of lumichrome as a *Sinorhizobium* enhancer of alfalfa root respiration and shoot growth Proc. Natl. Acad. Sci. USA 96, 12275–12280.

Poly F, Monrozier L J and Bally R 2001 Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Res. Microbiol. 152, 95–103.

Powledge F 1996 Making the Most of *Musa*. The role of the International Network for the Improvement of Banana and Plantain. *In* International Network for the Improvement of Banana and Plantain. Eds C Picq and R Raymond. INIBAP, International Plant Genetic Resources Institute, Montpellier, France.

Reinhold B, Hurek T, Niemann E -G and Fendrik I 1986 Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. Appl. Environ. Microbiol. 52, 520–526.

Reinhold-Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K and DeLey J 1993 *Azoarcus* gen nov., nitrogen-fixing Proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. 43, 574–584.

Robles-González M M and Orozco-Romero J 1996 Producción de plátano enano gigante mediante la técnica de cultivo de tejidos. Editorial Conexión Gráfica, S. A. de C. V. Guadalajara, México.

Rodríguez Cáceres E A 1982 Improved medium for isolation of *Azospirillum* spp. Appl. Environ. Microbiol. 44, 990–991.

Rogel M A, Hernández-Lucas I, Kuykendall L D, Balkwill D L and Martínez-Romero E 2001 Nitrogen-fixing nodules with *Ensifer adhaerens* harboring *Rhizobium tropici* symbiotic plasmids. Appl. Environ. Microbiol. 67, 3264–3268.

Selander R K, Caugant D A, Ochman H, Musser J M, Gilmour M N and Whittam T S 1986 Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl. Environ. Microbiol. 51, 873–884.

Sturz A V, Christie B R, Matheson B G and Nowak J 1997 Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biol. Fertil Soils 25, 13–19.

Tien T, Gaskins M H and Hubbell D H 1979 Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl. Environ. Microbiol. 37, 1016–1024.

Toledo I, Lloret L and Martínez-Romero E 2003 *Sinorhizobium americanum* sp nov., a new *sinorhizobium* species nodulating native *Acacia* spp. in Mexico. System Appl. Microbiol. 26, 54–64.

Wang E T, Rogel M A, García-de los Santos A, Martínez-Romero J, Cevallos M A and Martínez-Romero E 1999 *Rhizobium etli* bv. mimosae, a novel biovar isolated from *Mimosa affinis*. Int. J. Syst. Bacteriol. 49, 1479–1491.

Weber O B, Baldani J I and Döbereiner J 2000 Bactérias diazotróficas em mudas de bananeira. Pesquisa Agropecuária Brasileira. 35, 2227–2285.

Weber O B, Baldani V L D, Teixeira K R S, Kirchhof G, Baldani J I and Dobereiner J 1999 Isolation and characterization of diazotrophic bacteria from banana and pineapple plants. Plant Soil 210, 103–113.

Weber O B, Cruz L M, Baldani J I and Döbereiner J 2001 *Herbaspirillum*-like bacteria in banana plants. Braz. J. Microbiol. 32, 201–205.

Weisburg W G, Barns S M, Pelletier D A and Lane D J 1991 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

Xi C, Dirix G, Hofkens J, De Schryver F C, Vanderleyden J and Michiels J 2001 Use of dual marker transposons to identify new symbiosis genes in *Rhizobium*. Microbial. Ecol. 41, 325–332.

Yanni Y G, Rizk R Y, Abd El-Fattah F K, Squartini A, Corich V, Giacomini A, De Bruijn F, Rademaker J, Maya-Flores J and Ostrom P 2001 The beneficial plant growth-promoting association

of *Rhizobium leguminosarum* bv. trifolii with rice roots. Austr. J. Plant Physiol. 28, 845–870.

Yanni Y G, Rizk R Y, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S, Orgambide G, De Bruijn F, Stoltzfus J, Buckley D, Schmidt T M, Mateos P F, Ladha J K and Dazzo F B 1997 Natural endophytic association between *Rhizobium leguminosarum* bv. trifolii and rice roots and assessment of its potential to promote rice growth. Plant Soil 194, 99–114.

Young J M, Kuykendall L D, Martínez-Romero E, Kerr A and Sawada H 2001 A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn, 1942 and *Allorhizobium undicola* de Lajudie et al. (1998) as new combinations: *Rhizobium radiobacter*, *R. rhizogenes, R. rubi*, *R. undicola* and *R. vitis*. Int. J. Syst. Evol. Microbiol. 51, 89–103.