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# *Coffea arabica* L., a New Host Plant for *Acetobacter diazotrophicus*, and Isolation of Other Nitrogen-Fixing Acetobacteria

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**_Acetobacter diazotrophicus_ was isolated from coffee plant tissues and from rhizosphere soils. Isolation frequencies ranged from 15 to 40% and were dependent on soil pH. Attempts to isolate this bacterial species from coffee fruit, from inside vesicular-arbuscular mycorrhizal fungi spores, or from mealybugs (_Planococcus citri_) associated with coffee plants were not successful. Other acid-producing diazotrophic bacteria were recovered with frequencies of 20% from the coffee rhizosphere. These $N_2$-fixing isolates had some features in common with the genus _Acetobacter_ but should not be assigned to the species _Acetobacter diazotrophicus_ because they differed from _A. diazotrophicus_ in morphological and biochemical traits and were largely divergent in electrophoretic mobility patterns of metabolic enzymes at coefficients of genetic distance as high as 0.950. In addition, these $N_2$-fixing acetobacteria differed in the small-subunit rRNA restriction fragment length polymorphism patterns obtained with _Eco_RI, and they exhibited very low DNA-DNA homology levels, ranging from 11 to 15% with the _A. diazotrophicus_ reference strain PAl $5^T$. Thus, some of the diazotrophic acetobacteria recovered from the rhizosphere of coffee plants may be regarded as $N_2$-fixing species of the genus _Acetobacter_ other than _A. diazotrophicus_. Endophytic diazotrophic bacteria may be more prevalent than previously thought, and perhaps there are many more potentially beneficial $N_2$-fixing bacteria which can be isolated from other agronomically important crops.**

Almost 100 bacterial genera, of both the eubacteria and archaeobacteria, are capable of fixing $N_2$ (32). There may exist many more bacterial species or genera which can fix nitrogen since a majority of bacterial species are not presently culturable (31) and the search for diazotrophs in some environments has been relatively limited. Research on $N_2$-fixing bacteria endophytically associated with sugarcane led to the description of *Acetobacter diazotrophicus*, which is the only known nitrogen-fixing species of acetic acid-producing bacteria (13, 29). Similarly, in the last few years, the genus *Azoarcus* and its various species were described (16, 33), most of them recovered from the roots of Kallar grass (24). These findings suggest that many other endophytic $N_2$-fixing species may not yet have been described.

Looking for well-known $N_2$-fixing species and for new diazotrophs associated with previously untested plants or from new environments may provide a better picture not only of the distribution of $N_2$-fixation ability among bacterial taxa but also of the distribution and diversity of $N_2$-fixing bacterial populations.

In this work, we report the natural occurrence of diazotrophic acetic acid-producing bacteria in the rhizosphere and in tissues from different cultivars of seed-propagated coffee plants (*Coffea arabica* L.). Microbiological, biochemical, and genetic tests showed that a majority of these bacteria belong to the species *A. diazotrophicus*. We obtained evidence that strongly supports the hypothesis that some of the strains could represent new $N_2$-fixing species of the genus *Acetobacter.*

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## MATERIALS AND METHODS

**Locations and coffee cultivars.** Coffee plant varieties grown in nurseries or under field conditions were collected from diverse geographic regions of Mexico up to 750 km apart. The origins of samples and the coffee varieties analyzed are summarized in Table 1.

**Media and cultural conditions.** N-free semisolid LGI medium supplemented with sugarcane juice at pH 4.5 (7) and cycloheximide (150 mg/liter) was used for enrichment culturing of $N_2$-fixing acetobacters. For isolation and culturing, acetic acid LGI agar plates supplemented with yeast extract (50 mg/liter) and cycloheximide (150 mg/liter) and potato agar plates with 10% cane sugar were used (7). $N_2$-fixing acetobacters were grown at 29°C in SYP medium (6) for all other assays.

**Isolation.** Care was taken to keep rhizosphere soil intact around the root. Later, the root samples were rinsed three times in sterile distilled water. Separately, coffee root and stem pieces were immersed in 1% chloramine T for 5 min and treated as described previously (11). Root and stem samples were macerated in a blender, and supernatant aliquots (100 μl) were placed in vials containing 5 ml of N-free semisolid LGI medium (7). Other vials were inoculated with 100-μl aliquots from a 1/10 (wt/vol) rhizosphere soil suspension. Also, five samples (10 g each) of ripening fruit from *Coffea arabica* cv. Garnica collected in the coffee-growing region of Huitzilan, Puebla State, Mexico, were surface sterilized and treated as mentioned above for root and stem samples. In attempts to recover *A. diazotrophicus* from inside vesicular-arbuscular mycorrhizal (VAM) fungal spores, 100 g of eight rhizosphere soil samples (four from Huitzilan and four from Tapachula, Chiapas, Mexico) was sieved and at least 60 VAM spores were isolated from each soil sample by the method described by Gerdemann and Nicolson (12). The VAM spores were surface sterilized with 1% chloramine T for 5 min and then washed four times with sterile distilled water. Spores without apparent damage were manually crushed and placed in vials containing N-free semisolid LGI medium as reported previously (23). In addition, 50 adult mealybugs identified as *Planococcus citri* were analyzed for $N_2$-fixing acetobacters. These were collected from aerial parts of coffee plants, cultivar Caturra, growing in fields at Atoyac, Guerrero State, Mexico. Groups of 10 insects were rinsed with 0.01% (vol/vol) Tween 20 in 10 mM $MgSO_4 \cdot 7H_2O$ until the liquid was clear. Insects were macerated in 1.0 ml of 10 mM $MgSO_4 \cdot 7H_2O$, and 100-μl

TABLE 1. Isolation frequencies of *A. diazotrophicus* recovered from coffee plant cultivars

| Location | Cultivar | Plant age | pH of soil | Isolation (%) | | |
|---|---|---|---|---|---|---|
| | | | | Rhzp[a] | Root | Stem |
| Huitzilan, Puebla | Garnica | 5 yr[b] | 4.07 | 40.0 | 40.0 | 0.0 |
| | Garnica | 2 yr[b] | 6.27 | 0.0 | 0.0 | 0.0 |
| Xicotepec, Puebla | Catuai | 2 mo[c] | 4.74 | ND[d] | 0.0 | 20.0 |
| | Catuai | 6 mo[b] | 4.00 | ND | 20.0 | 0.0 |
| Atoyac, Guerrero | Caturra | 1 yr[b] | 3.64 | 15.0 | 20.0 | 0.0 |
| | M. Novo | 1 yr[c] | 6.20 | 0.0 | 0.0 | 0.0 |
| Tapachula, Chiapas | Caturra | 5 mo[c] | 5.40 | 30.0 | 0.0 | 0.0 |
| | Caturra | 5 mo[c] | 5.80 | 20.0 | 0.0 | 0.0 |
| | Caturra | 3 mo[c] | 5.30 | 40.0 | 0.0 | 0.0 |

[a] Rhzp, rhizosphere (soil shaken off roots).
[b] Coffee plants growing under field conditions.
[c] Coffee plants growing in a nursery.
[d] ND, not determined.

aliquots were inoculated into media for isolation of *A. diazotrophicus* as described previously (5).

Vials of inoculated N-free semisolid LGI medium were incubated at 30°C for 7 days. Thereafter, vials were replicated under the same conditions and assayed for acetylene reduction activity as described previously (21). Nitrogenase-positive vials with a yellow surface pellicle were streaked onto acetic LGI agar plates and incubated at 30°C. After 5 to 7 days, acid-producing dark-orange colonies suggested the presence of *A. diazotrophicus* (7). Colonies were streaked on potato agar plates to verify culture purity. In addition, atypical acid-producing isolates (referred to in the text as DOR and APL isolates) were also recovered from coffee rhizosphere samples from Tapachula. These isolates did not exhibit growth typical of *A. diazotrophicus* on LGI agar plates. DOR isolates were similar in their dark-orange color but formed very irregular smooth flat colonies. In addition, while *A. diazotrophicus* colonies are initially white and later become yellow-orange, DOR isolates are always orange. APL isolates showed a liquid-like appearance on the first days, but after 5 days, the isolates became dry and took on a yellowish color. One non-acid-producing mucoid strain (designated CFN-Cf 56) was also isolated due to its predominant growth on an LGI agar plate. This strain was selected based on its colony morphology, which was similar to that of a spontaneous, non-acid-producing mutant that was obtained from *A. diazotrophicus* SRT4 (1).

**Identification.** Isolate identification was based on colony morphology in culture media, on biochemical tests, and on genetic characteristics reported for *A. diazotrophicus* (5–7, 13). *A. diazotrophicus* PAl 5$^T$ (ATCC 49037), kindly provided by J. Döbereiner, and UAP 5560, analyzed previously (6, 11), were used as controls.

**MLEE.** Each isolate was grown for 36 h in 40 ml of SYP medium and harvested by centrifugation; pellets were suspended and treated as described previously (6). Starch gel electrophoresis and selective staining of metabolic enzymes were done as described before (25). The analyzed enzymes were the same ones used in a previous study (5) and were assayed under the same conditions. Distinct combinations of alleles for 12 enzyme loci (multilocus genotypes) were designated as different electrophoretic types (ETs) (25). *A. diazotrophicus* strains (CFNE 501, CFNE 550, PAl 5$^T$, PAl 3, 1772, PSP 22, and PRC 1), corresponding to the reported seven ETs (5), were included as references in multilocus enzyme electrophoresis (MLEE) assays to determine the genetic relationships of coffee plant-associated isolates and *A. diazotrophicus.*

**Total DNA isolation, DNA restriction, and filter blot hybridization.** Total DNA was isolated as described previously by Ausubel et al. (3). DNA was digested with *Eco*RI, and restriction fragments were electrophoresed in vertical 1.0% agarose gels in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8]) at 40V for 13 h at 4°C. Total DNA digests were transferred from gels to nylon filters by the Southern procedure as described before (6). The restriction fragment length polymorphism (RFLP) patterns of the *nifHDK* genes were determined by hybridization with a *Hin*dIII-*Hin*dIII 4.3-kb fragment containing the *nifHDK* genes from *A. diazotrophicus* UAP 5560 obtained from pUC19 derivative pNHAd4 (unpublished results). DNA-DNA homology was based on relative levels of hybridization to $^{32}$P-labelled DNA from strain PAl 5$^T$. Amounts of DNA in gels were quantified as described before (5). Autoradiography was performed at −70°C for 24 h; filter lanes were cut and counted with a Beckman scintillation counter. The percentage of total hybridization was calculated for each strain tested. Hybridization patterns of small-subunit (SSU) ribosomal DNA (rDNA) genes were analyzed as described before (5), but in this study, total DNA also was digested with restriction enzymes *Sph*I and *Nco*I. Genomic DNA from coffee plant-associated isolates and from strains PAl 5$^T$ and UAP 5560 of *A. diazotrophicus* were hybridized with an *Escherichia coli* SSU rRNA gene internal fragment from vector pKK3535 (4) corresponding to nucleotides 80 to 653. $^{32}$P-labelled probes were prepared by nick translation.

**SSU rDNA sequence alignment.** To search discriminative restriction sites in the SSU rRNA genes for distinguishing *Acetobacter* from other bacteria, we aligned 11 reported SSU rDNA sequences of different strains of the family *Acetobacteraceae* and 29 sequences of strains from other members of the α subclass of the class *Proteobacteria* (α-*Proteobacteria*) with GCG software version 8.1-UNIX (Genetics Computer Group, Madison, Wis.). GenBank accession numbers for SSU rDNA sequences aligned are shown in Table 2.

## RESULTS

**Isolation.** Typical yellow surface pellicles of nitrogen-fixing *Acetobacter* were observed in N-free LGI medium vials inoculated with rhizosphere soil, blended roots, and stems from different coffee plant varieties grown in various geographical areas of Mexico. On LGI agar plates, dark-orange colonies typical of *A. diazotrophicus* were observed (Fig. 1). Isolation frequencies from the rhizosphere, inside of roots, or stems ranged from 15 to 40% in plants grown in acid soils (Table 1). Additionally, from some rhizosphere samples, we recovered acid-producing DOR and APL isolates from LGI medium vials

TABLE 2. GenBank accession numbers used for the SSU rDNA sequence alignments

| Species | Strain | Accession no. |
|---|---|---|
| *Acetobacter pasteurianus* | LMD 22.1 | X71863 |
| *Acetobacter aceti* | DSM 3508 | X74066 |
| *Acetobacter liquefaciens* | LMG 1382 | X75617 |
| *Acetobacter diazotrophicus* | PAl 5$^T$ | X75618 |
| *Acetobacter xylinum* | NCIB 11664 | X75619 |
| *Acetobacter hansenii* | NCIB 8746 | X75620 |
| *Acetobacter europaeus* | DSM 6160 | Z21936 |
| *Gluconobacter oxydans* | DSM 3503 | X73820 |
| *Gluconobacter asaii* | LMG 1390 | X80165 |
| *Gluconobacter cerinus* | LMG 1368 | X80775 |
| *Gluconobacter frateurii* | LMG 1365 | X82290 |
| *Acidomonas methanolica* | LMG 1668[a] | X77468 |
| *Acidiphilium* sp. | C-1 | D30769 |
| *Acidiphilium aminolytica* | 101 | D30771 |
| *Acidiphilium angustum* | ATCC 35903 | D30772 |
| *Acidiphilium cryptum* | ATCC 33463 | D30773 |
| *Acidiphilium facilis* | ATCC 35904 | D30774 |
| *Acidiphilium organovorum* | ATCC 43141 | D30775 |
| *Acidiphilium rubrum* | ATCC 35905 | D30776 |
| *Acidiphilium* sp. | St 1-5 | D86508 |
| *Acidiphilium* sp. | St 1-7 | D86509 |
| *Rhodopila globiformis* | DSM 161 | D86513 |
| *Rhodopila globiformis* | ATCC 7950 | M59066 |
| *Rhodospirillum* sp. | MT-SP-3 | D12703 |
| *Rhizobium meliloti* | IAM12611 | D14509 |
| *Rhizobium leguminosarum* | IAM12609 | D14513 |
| *Rhodopseudomonas* sp. | IL-245 | D15063 |
| *Rhodobacter capsulatus* | ATCC 11166 | D16428 |
| *Rhodospirillum rubrum* | ATCC 11170 | D30778 |
| *Beijerinckia indica* | ATCC 9039 | M59060 |
| *Caulobacter* sp. | MCS 6 | M83811 |
| *Hyphomonas* sp. | MHS 3 | M83812 |
| *Hyphomicrobium vulgare* | MC-750 | X53182 |
| *Roseobacter litoralis* | ATCC 49556 | X78312 |
| *Azospirillum lipoferum* | ATCC 29708 | X79729 |
| *Azospirillum irakense* | 103312 | X79737 |
| *Azospirillum brasilense* | Sp 7 | X79739 |
| *Azospirillum amazonense* | Y2 | X79742 |
| *Xanthobacter flavus* | JW/KR-E1 | X94206 |
| *Pedomicrobium manganicum* | ACM 3038 | X97691 |

[a] Substrain MB 58.

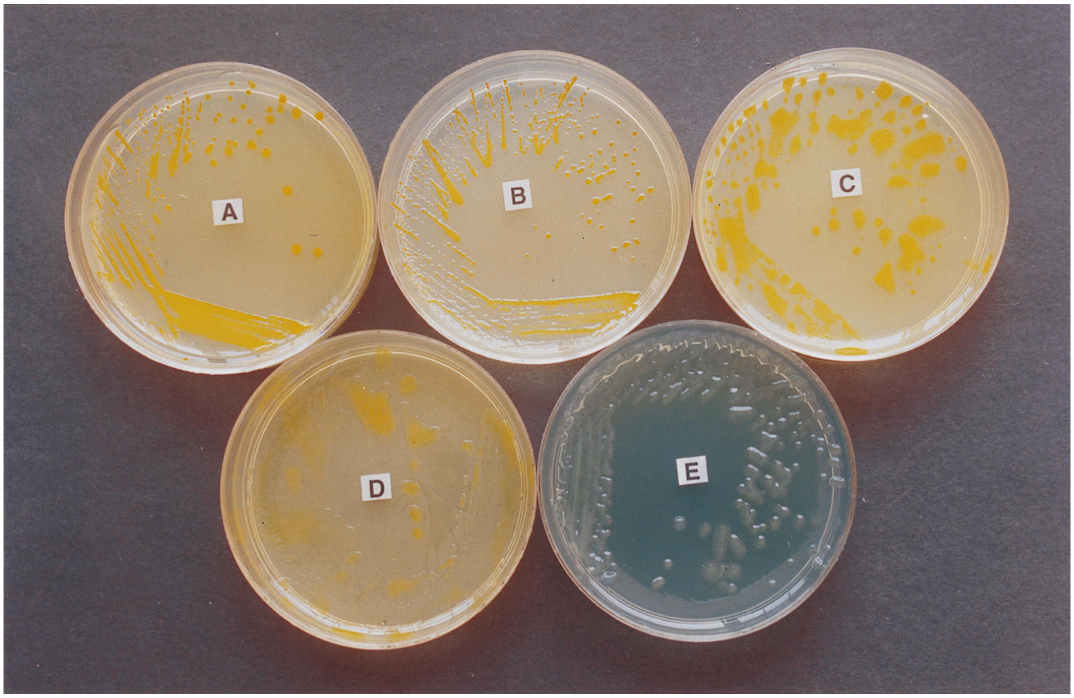


FIG. 1. Colony morphologies of $N_2$-fixing acetobacters after 7 days at 29°C on LGI agar plates. (A and B) *A. diazotrophicus* PAl $5^T$ (A) and CFN-Cf 50 (B); (C) DOR isolate, strain CFN-Cf 55; (D) APL isolate, strain UAP-Cf 60; (E) mucoid strain CFN-Cf 56. The green color in LGI agar plates was turned to yellow by acid-producing isolates.

with yellow surface pellicles. These isolates reduced acetylene in pure culture but had clearly different morphologies from that of *A. diazotrophicus* on LGI agar plates (Fig. 1). These DOR and APL isolates were recovered from two rhizosphere samples (collected in Tapachula) with isolation frequencies of 20%. Strain CFN-Cf 56, which does not produce acid on LGI agar plates, was the only mucoid isolate recovered (Fig. 1).

No bacteria corresponding to the descriptions given above were isolated from coffee plants growing at a pH higher than 6.0 nor from coffee fruit, VAM spores, or mealybugs (*P. citri*).

**MLEE and genetic relationships.** The origins of the coffee-associated $N_2$-fixing isolates are shown in Table 3. The genetic relationships among the $N_2$-fixing isolates associated with coffee plants and *A. diazotrophicus* strains recovered from known hosts are illustrated by the dendrogram shown in Fig. 2. Thirteen distinct ETs were identified among $N_2$-fixing coffee iso-

TABLE 3. Origins of representative $N_2$-fixing bacteria recovered from the coffee environment

| MLEE division (ET)[a] | Type of isolate | Strain designation | Isolate recovered from: | Plant age | Cultivar | Location |
|---|---|---|---|---|---|---|
| I (1) | *A. diazotrophicus* | CFN-Cf13 | Stem tissue | 2 mo[b] | Catuai | Xicotepec, Puebla |
| I (11) | *A. diazotrophicus* | CFN-Cf50 | Root tissue | 6 mo[c] | Catuai | Xicotepec, Puebla |
| I (1) | *A. diazotrophicus* | UAP-Cf29 | Rhizosphere | 1 yr[c] | Caturra | Atoyac, Guerrero |
| I (9) | *A. diazotrophicus* | CFN-Cf52 | Root tissue | 1 yr[c] | Caturra | Atoyac, Guerrero |
| I (8) | *A. diazotrophicus* | UAP-Cf01 | Rhizosphere | 5 yr[c] | Garnica | Huitzilan, Puebla |
| I (8) | *A. diazotrophicus* | UAP-Cf05 | Root tissue | 5 yr[c] | Garnica | Huitzilan, Puebla |
| I (12) | *A. diazotrophicus* | UAP-Cf51 | Rhizosphere | 5 mo[b] | Caturra | Tapachula, Chiapas |
| I (14) | *A. diazotrophicus* | UAP-Cf53 | Rhizosphere | 3 mo[b] | Caturra | Tapachula, Chiapas |
| I (10) | *A. diazotrophicus* | UAP-Cf58 | Rhizosphere | 5 mo[b] | Caturra | Tapachula, Chiapas |
| I (13) | NAP[d] | CFN-Cf56 | Rhizosphere | 3 mo[b] | Caturra | Tapachula, Chiapas |
| III (15) | APL[e] | UAP-Cf59 | Rhizosphere | 5 mo[b] | Caturra | Tapachula, Chiapas |
| III (16) | APL | CFN-Cf60 | Rhizosphere | 3 mo[b] | Caturra | Tapachula, Chiapas |
| IV (17) | DOR[f] | CFN-Cf55 | Rhizosphere | 3 mo[b] | Caturra | Tapachula, Chiapas |
| IV (18) | DOR | UAP-Cf57 | Rhizosphere | 5 mo[b] | Caturra | Tapachula, Chiapas |
| V (19) | SAd[g] | CFN-Cf54 | Rhizosphere | 5 mo[b] | Caturra | Tapachula, Chiapas |

[a] Divisions and ETs were based on MLEE assays. More isolates included in divisions I and III to V were recovered, but only one of the many isolates recovered from each plant or rhizosphere sample was designated as a strain.
[b] Coffee plants growing in a nursery.
[c] Coffee plants growing under field conditions.
[d] NAP, non-acid-producing isolate.
[e] APL, acid-producing liquid isolate.
[f] DOR, dark-orange isolate.
[g] SAd, isolate with colonial features similar to those of *A. diazotrophicus.*

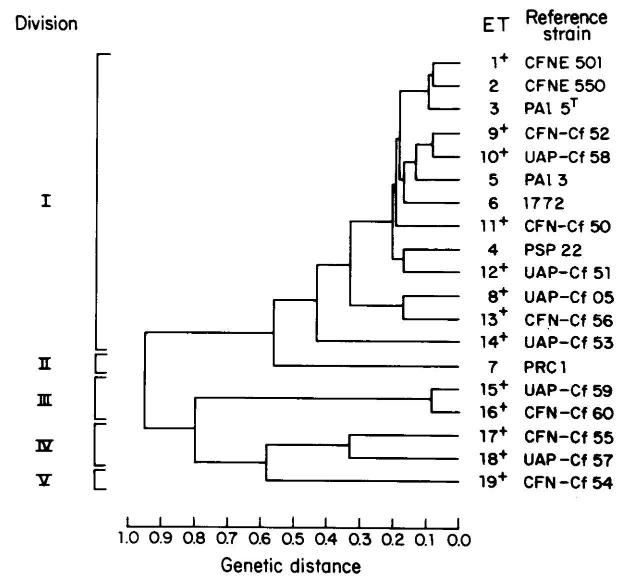


FIG. 2. Genetic relationships of ETs identified among *A. diazotrophicus* isolates recovered from well-known hosts and $N_2$-fixing acetobacters associated with coffee plants. A plus after the ET number indicates that the ET represents only coffee plant-associated nitrogen-fixing acetobacters, except for ET 1, which includes reported reference strains as well.

lates (multilocus genotype data are available upon request). Division I, with a genetic distance of 0.430, included six previously identified ETs (ET 1 to ET 6) (5) and six new closely related ETs (ET 8 to ET 12 and ET 14) from coffee-associated *A. diazotrophicus* isolates. In addition, division I included ET 13, which corresponds to an isolate (CFN-Cf 56) with no typical features of *A. diazotrophicus*. Moreover, isolates recovered from both the rhizosphere (e.g., strain UAP-Cf 29) and the inside of coffee plants (e.g., strain CFN-Cf 13) were identical to strains of *A. diazotrophicus* belonging to ET 1, previously identified (5, 6) as the predominant ET (e.g., UAP 5560 and CFNE 501) of the species. Division II contained only ET 7, a genetically distant group previously identified (5) among *A. diazotrophicus* strains isolated from sugarcane and *Pennisetum purpureum* in Brazil. Divisions III, IV, and V, which included ETs 15 to 19, diverged largely at a genetic distance of 0.950 from divisions I and II. Division III (ETs 15 and 16) contained only APL isolates, while division IV (ETs 17 and 18) included DOR isolates and division V (ET 19) grouped isolates with colonial features similar to those of *A. diazotrophicus* on acetic LGI agar plates.

**Identification.** Many isolates recovered from the inside of coffee plants and from the rhizosphere of these plants were identified as belonging to the species *A. diazotrophicus* on the basis of reported characteristics (5, 6, 7, 13) such as growth features on culture media, biochemical tests, and results of genetic approaches (Tables 4 and 5). Other isolates such as the mucoid strain CFN-Cf 56 and the DOR and APL strains differed from *A. diazotrophicus* in various phenotypic characteristics (Table 4 and carbon usage data not shown). Nevertheless, these isolates were able to grow at pH 5, oxidize ethanol to acetic acid in neutral and acid conditions, and oxidize acetate and lactate to $CO_2$ and $H_2O$ (Table 4), phenotypic features which are considered (8, 29) fundamental for the identification of the genus *Acetobacter.*

**Genetic characteristics.** Total *Eco*RI DNA digests from coffee isolates, including those with different colony morpholo-

TABLE 4. Phenotypic characteristics of $N_2$-fixing acetic acid bacteria isolated from the coffee plant environment[a]

| Characteristic | I[b] | | | | | | III | | IV | | V |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PAI 5[Tc] | UAP 5560[c] | CFN-Cf 13 | UAP-Cf 05 | CFN-Cf 52 | CFN-Cf 56 | UAP-Cf 59 | CFN-Cf 60 | CFN-Cf 55 | UAP-Cf 57 | CFN-Cf 54 |
| Gram stain | − | − | − | − | − | − | − | − | − | − | − |
| Oxidase | − | − | − | − | − | − | − | − | − | − | − |
| Catalase | + | + | + | + | + | + | + | + | + | + | + |
| Oxidation of ethanol to acetic acid | + | + | + | + | + | +[d] | + | + | + | + | + |
| Oxidation of glucose to acetic acid | + | + | + | + | + | + | + | + | + | + | + |
| Oxidation of acetic acid to $CO_2$ and $H_2O$ | + | + | + | + | + | + | + | + | + | + | + |
| Oxidation of lactate to $CO_2$ and $H_2O$ | + | + | + | + | + | + | + | + | + | + | + |
| Water-soluble brown pigments on GYC[f] | + | + | + | + | − | − | − | − | + | + | + |
| Dark-orange colonies on LGI plates | + | + | + | + | + | − | − | − | + | + | + |
| Dark-brown colonies on potato agar with 10% sugar | + | + | + | + | + | − | − | − | − | − | − |
| Brownish colonies on potato agar with 10% sugar | − | − | − | − | − | − | −[e] | −[e] | + | + | + |
| Growth with 30% D-glucose | + | + | + | + | + | + | + | + | + | + | + |
| Growth with 30% sucrose | + | + | + | + | + | + | + | + | + | + | + |
| Yellow surface pellicle formation and pH below 3 in N-free semisolid LGI medium | + | + | + | + | + | − | + | + | + | + | + |
| $C_2H_2$ reduction activity ($N_2$ fixation) | + | + | + | + | + | + | + | + | + | + | + |

[a] Phenotypic characteristics were positive (+) or negative (−) for each strain.
[b] Divisions based on MLEE assays.
[c] *A. diazotrophicus* strains recovered from sugarcane used as controls.
[d] Oxidation was observed up to day 4.
[e] Cream-colored colonies, but producing a brownish liquid pigment.
[f] GYC, 5% D-glucose–1% yeast extract–3% $CaCO_3$–2.5% agar (8).

TABLE 5. Genetic characteristics of some $N_2$-fixing acetobacters recovered from the coffee plant environment[a]

| MLEE division[a] (ET)[a] | Type of isolate[b] | Reference strain | Sizes (kb)[c] | | DNA-DNA homology (%)[d] |
|---|---|---|---|---|---|
| | | | *nifHDK* genes | SSU rDNA genes | |
| I (3) | *A. diazotrophicus* | PAl 5[Te] | 9.0, 2.0, 1.25 | 9.3, 3.6, 2.3, 1.6 | 100.0 |
| I (1) | *A. diazotrophicus* | UAP 5560[e] | 9.0, 2.0, 1.25 | 9.3, 3.6, 2.3, 1.6 | 104.0 |
| I (8) | *A. diazotrophicus* | UAP-Cf 05 | 9.0, 3.1, 1.25 | 9.3, 3.6, 2.3, 1.6 | ND[f] |
| I (9) | *A. diazotrophicus* | CFN-Cf 52 | 9.0, 2.0, 1.25 | 9.3, 3.6, 2.3, 1.6 | 83.0 |
| I (10) | *A. diazotrophicus* | UAP-Cf 58 | 9.0, 2.0, 1.25 | 9.3, 3.6, 2.3, 1.6 | 78.0 |
| I (11) | *A. diazotrophicus* | CFN-Cf 50 | 9.0, 2.0, 1.25 | 9.3, 3.6, 2.3, 1.6 | 96.0 |
| I (12) | *A. diazotrophicus* | UAP-Cf 51 | 9.0, 2.0, 1.25 | 9.3, 3.6, 2.3, 1.6 | 72.0 |
| I (14) | *A. diazotrophicus* | UAP-Cf 53 | 9.0, 2.0, 1.25 | 9.3, 3.6, 2.3, 1.6 | 77.0 |
| I (13) | NAP | CFN-Cf 56 | 7.6, 3.5, 1.20, 1.0 | 9.3, 3.6, 2.3, 1.6 | 30.0 |
| III (15) | APL | UAP-Cf 59 | Not detected | 8.6, 3.9, 3.6, 1.6 | 12.0 |
| III (16) | APL | CFN-Cf 60 | Not detected | 8.6, 3.9, 3.6, 1.6 | 15.0 |
| IV (17) | DOR | CFN-Cf 55 | 9.0, 2.0, 1.20 | 9.7, 3.6, 1.6 | 14.0 |
| IV (18) | DOR | UAP-Cf 57 | 9.0, 2.0, 1.20 | 9.7, 3.6, 1.6 | 15.0 |
| V (19) | SAd | CFN-Cf 54 | 6.6, 2.1, 1.15 | 9.7, 3.6, 2.8, 1.6 | 11.0 |

[a] Divisions and ETs were based on MLEE assays.
[b] Types described in Table 3, footnotes *d*, *e*, *f*, and *g*.
[c] Bands from total *Eco*RI DNA fingerprints hybridized as described in Materials and Methods.
[d] Homology to the control strain PAl 5[T].
[e] *A. diazotrophicus* strains recovered from sugarcane used as controls.
[f] ND, not determined.

gies, were hybridized to *A. diazotrophicus nifHDK* genes (Fig. 3). Three common hybridizing bands were observed for representative isolates of the 6 ETs from division I (Table 5), as reported previously (5, 6). In addition, isolates represented by strain UAP-Cf 05 (division I) and isolates grouped in division IV (e.g., CFN-Cf 55 and UAP-Cf 57) showed bands that differed from each other slightly in size (Table 5). Strain CFN-Cf 54 (division V) and the mucoid strain CFN-Cf 56 showed a more variable pattern of the *nifHDK* genes. No hybridizing bands were observed under stringent hybridization conditions with APL strains from division III (Fig. 3), even though pure cultures of these isolates were capable of reducing acetylene. This result seems to indicate that structural nitrogenase genes from APL isolates are largely divergent from *A. diazotrophicus nifHDK* genes.

RFLP analysis of *Eco*RI DNA digests from coffee plant-associated isolates showed four distinct hybridization patterns to SSU rRNA genes (Fig. 4). Among the patterns obtained, two common hybridizing bands (3.6 and 1.6 kb) were observed. All isolates of division I showed the same pattern of hybridization (Table 5) as that observed previously in all *A. diazotrophicus* strains analyzed (5). $N_2$-fixing *Acetobacter* strains diverging at a large genetic distance from divisions I and II, according to the MLEE assays, presented different SSU rRNA hybridization patterns (Fig. 4 and Table 5). Isolates grouped in division IV did not have the 2.3-kb band which seems to correspond to the 3.9- and 2.8-kb bands observed in the strains from divisions III and V, respectively.

From the SSU rDNA sequence analysis, we inferred that Southern hybridization with a SSU rDNA probe of *Sph*I-digested genomic DNA could be helpful in distinguishing members of the family *Acetobacteraceae* from other α-*Proteobacteria* (Fig. 5) and that *Nco*I digests could be used to distinguish the genera *Gluconobacter* and *Acetobacter* from *Acidiphilium* and *Rhodopila* (Fig. 5) (26). The majority of *Acetobacteraceae* spe-

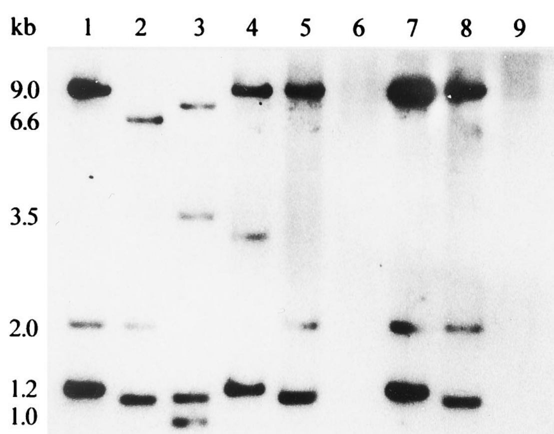


FIG. 3. Autoradiogram of a Southern blot of total *Eco*RI-digested DNA hybridized with the *nifHDK* probe of *A. diazotrophicus* UAP 5560. Lanes: 1, strain UAP 5560 used as a control; 2 to 9, coffee plant-associated nitrogen-fixing strains CFN-Cf 54 (lane 2), CFN-Cf 56 (lane 3), UAP-Cf 05 (lane 4), CFN-Cf 57 (lane 5), UAP-Cf 59 (lane 6), UAP-Cf 13 (lane 7), CFN-Cf 55 (lane 8), and CFN-Cf 60 (lane 9).

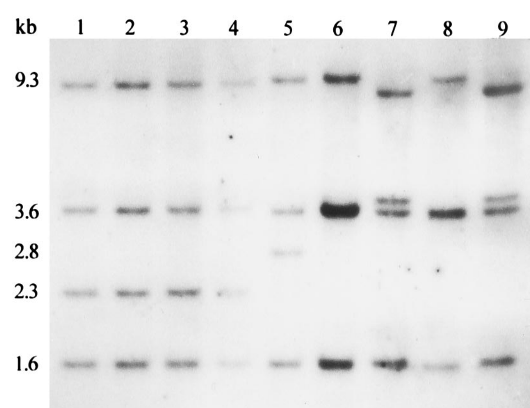


FIG. 4. Autoradiogram of a Southern blot of total *Eco*RI-digested DNA hybridized with an internal 16S rDNA probe of *E. coli*. Lanes: 1, strain UAP 5560 used as a control; 2 to 9, coffee-associated nitrogen-fixing strains UAP-Cf 13 (lane 2), UAP-Cf 05 (lane 3), CFN-Cf 56 (lane 4), CFN-Cf 54 (lane 5), UAP-Cf 57 (lane 6), UAP-Cf 59 (lane 7), CFN-Cf 55 (lane 8), and CFN-Cf 60 (lane 9).

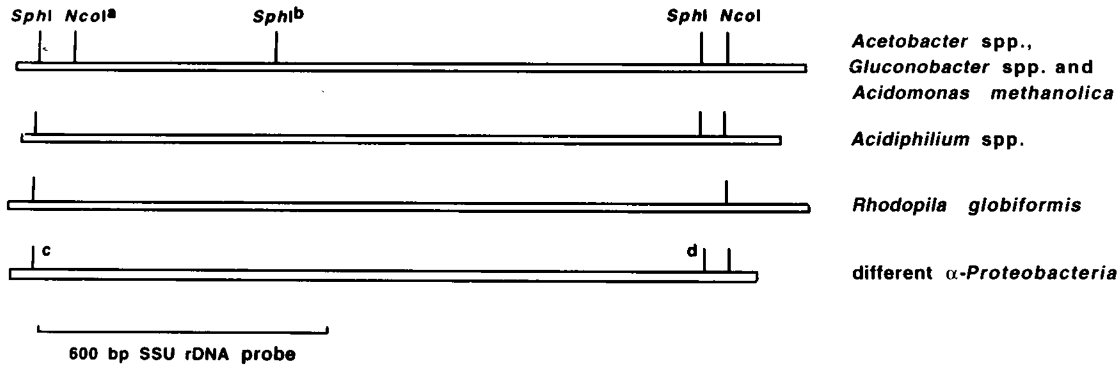


FIG. 5. Diagrammatic representation of distinctive restriction sites *Sph*I and *Nco*I of SSU rRNA in *Acetobacteraceae* and phenotypically related bacteria. a, site not present in *G. oxydans* DSM 3503; b, site exclusively present in the *A. diazotrophicus* PAl $5^T$ sequence but not detected after Southern hybridization; c, site present in *Azospirillum lipoferum* ATCC 29708 and *Azospirillum amazonense* Y2; d, of 17 analyzed sequences, this site exclusively present in *Rhizobium meliloti* IAM 12611, *Rhizobium leguminosarum* IAM 12609, *Caulobacter* sp. strain MCS 6, *Hyphomonas* sp. strain MHS 3, and *Xanthobacter flavus* JW/KR-E1.

cies, including *Acidomonas methanolica*, have two internal *Sph*I sites in their SSU rDNA, except for *A. diazotrophicus* PAl $5^T$ (accession number X75618), which supposedly has an extra *Sph*I site at base 485 as deduced from the reported sequence (Fig. 5). *Rhodopila globiformis* (accession numbers D86513 and M59066) lacks one of the *Sph*I sites. From the analysis of the *A. diazotrophicus* PAl $5^T$ SSU rDNA sequence (26), we expected to observe one hybridizing band of 450 bp with the probe used when the DNA was digested with *Sph*I. However, only one SSU rRNA hybridizing band of 1.3 kb was observed in *A. diazotrophicus* PAl $5^T$ and UAP 5560. This band was conserved in all coffee plant-associated isolates. These conflicting results may be explained if the *A. diazotrophicus* sequence has an error at the *Sph*I site. If such were the case, then the *Acetobacteraceae* and *Acidiphilium* spp. would have only two *Sph*I conserved sites. *Gluconobacter* and *Acetobacter* SSU rDNA are characterized by two *Nco*I restriction sites. However, all *Acidiphilium* and *Rhodopila* species and *Gluconobacter oxydans* lack the *Nco*I restriction site at the base corresponding to nucleotide 110 of *A. diazotrophicus*. The rest of the α-*Proteobacteria* analyzed lack at least one site for each restriction enzyme. Genomic DNA from the strains recovered from the coffee plant environment, digested with *Nco*I and hybridized to the same SSU rRNA internal gene fragment, showed the expected 1.24-kb band (26) (data not shown).

The results of the DNA-DNA homology assays are shown in Table 5. The six strains of $N_2$-fixing acetobacters corresponding to division I (except strain CFN-Cf 56) analyzed were related to *A. diazotrophicus* PAl $5^T$ with DNA homology values of 72 to 96%, with a mean DNA homology of 81%. This value was consistent with the values of 86 and 84% reported previously (5, 13) for *A. diazotrophicus* strains recovered from sugarcane and other known hosts. The mucoid strain CFN-Cf 56 exhibited only 30% DNA homology to strain PAl $5^T$. APL isolates (MLEE division III) and DOR acetobacters from division IV and strains from division V exhibited very low DNA homology levels, ranging from 11 to 15% with reference strain PAl $5^T$.

## DISCUSSION

It is considered that "the isolation of acetic acid bacteria and their assignment to either the genus *Acetobacter* or *Gluconobacter* generally pose few problems" (29). According to Swings (29), gram-negative or gram-variable aerobic bacteria that oxidize ethanol to acetic acid in neutral or acid media are candidates for the family *Acetobacteraceae*. This family is divided into the genera *Gluconobacter*, which includes three species, and *Acetobacter*, in which seven species have been identified (29). Only the species *A. diazotrophicus* is capable of fixing $N_2$ (13). On the basis of these and other phenotypic features used for a satisfactory identification (29), we considered that the diazotrophic isolates recovered from the coffee plant environment belong to the family *Acetobacteraceae*. Phenotypic identification was confirmed by the SSU rRNA genes obtained with total DNA digested with *Nco*I and *Sph*I (data not shown). Moreover, we have considered it suitable to assign these $N_2$-fixing isolates to the genus *Acetobacter* because they were capable of oxidizing ethanol, first to acetic acid and then further to $CO_2$ and $H_2O$ (overoxidation of ethanol), which is the main feature of the genus (8, 29). Other differential phenotypic characteristics analyzed (Table 4) were in agreement with descriptions for this genus (8, 29). By taking into account the differential phenotypic features at the species level (8, 29) and with support from the MLEE assays and the molecular characteristics reported previously, such as hybridization patterns of *nifHDK* genes and of SSU rDNA genes (5, 6) as well as DNA-DNA homology experiments, a majority of the $N_2$-fixing *Acetobacter* isolates (all strains from division I, excluding CFN-Cf 56) recovered from rhizosphere soil and from inside tissues of coffee plants were considered to belong to the species *A. diazotrophicus*. Although *A. diazotrophicus* strains were reported to form water-soluble brown pigments on GYC medium (7), some of the *A. diazotrophicus* strains (CFN-Cf 52, UAP-Cf 51, Cf 53, and Cf 58) recovered from the coffee plant environment did not produce them (Table 4). However, water-soluble brown pigment production is not a typical feature of the genus *Acetobacter* but rather of the genus *Frateuria* (30). Thus, the *A. diazotrophicus* isolates not producing water-soluble brown pigments could be considered more typical acetobacters.

A number of *Acetobacter* isolates recovered from the coffee plant rhizosphere, capable of fixing $N_2$ under microaerobic conditions, should not be assigned to the species *A. diazotrophicus* because remarkable differences were observed. We propose that the strains corresponding to ETs included in divisions III, IV, and V may be regarded as different $N_2$-fixing species of the genus *Acetobacter*. This is based on the fact that all of these isolates were easily differentiated from *A. diazotrophicus* by several morphological and biochemical traits, including the electrophoretic mobility patterns of metabolic enzymes, rendering coefficients of genetic distance as high as 0.950.

Furthermore, these *Acetobacter* isolates differed in SSU rRNA RFLP patterns, and they had a very low level of DNA homology with *A. diazotrophicus* PAl $5^T$. These data are strong evidence to designate other diazotrophic species of the genus *Acetobacter*, but more $N_2$-fixing isolates from other coffee-producing areas of Mexico have to be isolated to provide an extended phenotypic and genetic analysis useful for taxonomic validation of a new species. This is specially true for strain CFN-Cf 56, which is a unique isolate with peculiar characteristics. For instance, on the basis of the MLEE data and SSU rRNA RFLP patterns, the strain CFN-Cf 56 should be regarded as belonging to the species *A. diazotrophicus*. However, on the basis of DNA-DNA homology values, this strain may be considered a new nitrogen-fixing species of the genus *Acetobacter*. Nevertheless, plasmid differences could account for the low DNA-DNA homology values between strain CFN-Cf 56 and strain PAl $5^T$.

Natural habitats of acetic acid bacteria are sugar and alcohol solutions, with flowers and many fruits being excellent habitats (29). *A. diazotrophicus*, an endophytic bacterial species, occurs predominantly in vegetatively propagated plants (9). It has been recovered from inside tissues of sucrose-accumulating plants such as sugarcane (10, 11, 19), from a few samples of washed roots and aerial parts of *Pennisetum purpureum* cv. Cameroon, and from sweet potato stems and roots (9) as well as from different genera of mealybugs associated with sugarcane plants (2, 5). This species has not been recovered from other plants nor from nonrhizosphere soils collected from sugarcane fields or other sites (9, 19). However, *A. diazotrophicus* was detected in sugarcane rhizosphere soil by the indirect enzyme-linked immunosorbent assay method (20).

In this study, *A. diazotrophicus* was isolated mainly from coffee plant rhizosphere soils but also, in lower frequencies, from surface-sterilized stems and roots of coffee plants. Our results strongly contrast those of previous reports in which *A. diazotrophicus* isolation from the sugarcane rhizosphere was a rare event. The occurrence of VAM fungus species associated with coffee plants (28) could explain the frequent isolation of *A. diazotrophicus* from the rhizosphere since this bacterial species has been reported to occur inside VAM fungal spores (23), and these were not discarded from the soil inoculated into the culture medium. However, our results did not support the former possibility because we were unable to recover *A. diazotrophicus* from VAM spores. The recovery of $N_2$-fixing acetobacters from the rhizosphere, we suspect, could be in relation to the organic matter content present in the rhizosphere of coffee plants. While sugarcane is burned off before cutting, eliminating virtually all organic matter originating both from senescent and trash leaves, in coffee-producing areas, the falling fruit and leaves of these trees are largely accumulated in the soil. Perhaps this organic matter could protect bacteria against soil physicochemical factors. In addition, the organic matter degradation by microbial communities will enrich the rhizosphere with carbon (sugar) sources usable by acetobacters. Contrasting with previous results, our data demonstrated that *A. diazotrophicus* is capable of colonizing plants propagated through seeds in addition to plants propagated vegetatively.

Clearly, the distribution of *A. diazotrophicus* is wider than early reports indicated. Genotype ET 1 is extensively distributed, not only among the previously reported hosts (5, 6) but also among coffee plant isolates. Perhaps ET 1 strains have a large colonization capacity that could be related to the presence of a highly conserved plasmid (pAd170) that exists in most ET 1 *A. diazotrophicus* isolates (6). This plasmid has not been observed in isolates corresponding to other ETs (6; unpublished results). pAd170 was also observed in ET 1 isolates recovered both from the rhizosphere and inside coffee plants (data not shown).

Coffee-associated genotypes, except ET 1, were never identified among more than 70 *A. diazotrophicus* strains recovered from previously well-known hosts collected in diverse countries (5, 6). Because isolates of *A. diazotrophicus* recovered from the coffee plant environment are closely related genetically to isolates recovered from sugarcane, the existence of a common lineage is suggested.

It is worth noting that even though the isolation of *A. diazotrophicus* from internal tissues was infrequent, it was usually recovered from coffee plants grown in acid soils. The infrequency of recovery of *A. diazotrophicus* from coffee plant tissues may be related to the difficulties in homogenizing roots and stems, since these plants are highly lignified and very hard to blend. The presence of *A. diazotrophicus* in acid soils suggests that the transmission of this species into coffee plants could be through VAM fungi, as reported for sugarcane plants (22) and *Sorghum bicolor* (17). Also, we considered that transmission of *A. diazotrophicus* could be through mealybugs, as suggested previously (2), or directly into coffee plant fruit, as occurs in pineapple with other acetic acid bacteria (15). Nevertheless, we were not able to recover *A. diazotrophicus* nor any other $N_2$-fixing acetobacters from coffee plant fruit or mealybugs (*Planococcus citri*). From these results, we may speculate that *A. diazotrophicus* uses root tips and cracks at lateral root junctions to enter the coffee plants, as suggested for sugarcane plants (18).

Our results support the hypothesis that in nature there are many more $N_2$-fixing bacteria to be identified and also strongly suggest that endophytic diazotrophic bacteria are more prevalent than previously was thought.

Considering the great economic importance of coffee in the world, and the difficulties of obtaining nitrogen fertilizers (14), we consider that coffee-associated $N_2$-fixing acetobacters may be agronomically important because they could supply part of the nitrogen that the crop requires, as has been suggested in the case of sugarcane with its associated endophytic nitrogen-fixing bacteria.

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