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

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Received 23 December 1996/Accepted 12 June 1997

*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 N₂-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 N₂-fixing acetobacteria differed in the small-subunit rRNA restriction fragment length polymorphism patterns obtained with EcoRI, and they exhibited very low DNA-DNA homology levels, ranging from 11 to 15% with the *A. diazotrophicus* reference strain PAI ⁵*. Thus, some of the diazotrophic acetobacteria recovered from the rhizosphere of coffee plants may be regarded as N₂-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 N₂-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 N₂ (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 N₂-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 N₂-fixing species may not yet have been described.

Looking for well-known N₂-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 N₂-fixation ability among bacterial taxa but also of the distribution and diversity of N₂-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 N₂-fixing species of the genus *Acetobacter*.

**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 N₂-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). N₂-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 N₂-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 MgSO₄·7H₂O until the liquid was clear. Insects were macerated in 1.0 ml of 10 mM MgSO₄·7H₂O and 100-μl
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DNA from coffee plant-associated isolates and from strains PAl 57 and UAP 5560 of A. diazotrophicus were hybridized with an Escherichia coli SSU rRNA gene internal fragment from vector pKK335 (4) corresponding to nucleotides 80 to 653. 32P-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>
<thead>
<tr>
<th>Location</th>
<th>Cultivar</th>
<th>Plant age</th>
<th>pH of soil</th>
<th>Rhiz*</th>
<th>Root</th>
<th>Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huitzilan, Puebla</td>
<td>Garnica 5 yr</td>
<td>4.07</td>
<td>40.0</td>
<td>40.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Garnica 2 yr</td>
<td>6.27</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Xicotepex, Puebla</td>
<td>Catuai 2 mo</td>
<td>4.74</td>
<td>ND*</td>
<td>0.0</td>
<td>20.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Catuai 6 mo</td>
<td>4.00</td>
<td>0.0</td>
<td>20.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Atoyac, Guerrero</td>
<td>Caturra 1 yr</td>
<td>3.64</td>
<td>15.0</td>
<td>20.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>M. Novo 1 yr</td>
<td>6.20</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Tapachula, Chiapas</td>
<td>Caturra 5 mo</td>
<td>5.40</td>
<td>30.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Caturra 5 mo</td>
<td>5.80</td>
<td>20.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Caturra 3 mo</td>
<td>5.30</td>
<td>40.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Rhizp, rhizosphere (soil shank off roots).
* Coffee plants growing under field conditions.
* Coffee plants growing in a nursery.
* ND, not determined.

Species Strain Accession no.

Acetobacter pasteurianus LMD 22.1 X71863
Acetobacter acetii DSM 3508 X74066
Acetobacter liquefaciens LMG 1382 X75617
Acetobacter diazotrophicus PAI 57 X75618
Acetobacter xylinum NCIB 11664 X75619
Acetobacter hansenii NCIB 8746 X75620
Acetobacter europaeus DSM 6160 Z21936
Glucobacter oxydan DSM 3503 X73820
Glucobacter asaizu DSM 1390 X80165
Glucobacter curvis DSM 1368 X80775
Glucobacter frateurii LMG 1755 X82290
Acidomonas methanolicus LMG 1668a X77468
Acidiphilium sp. C-1 D30769
Acidiphilium aminolytica 101 D30771
Acidiphilium angustum ATCC 35903 D30772
Acidiphilium cryptum ATCC 35346 D30773
Acidiphilium facilis ATCC 39504 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 X85613
Rhodopila globiformis ATCC 7950 M59066
Rhodopseudomonas sp. MT-SP-3 D12703
Rhizobium melliloti IAM12611 D14509
Rhizobium leguminosarum IAM12609 D14513
Rhizobium lupinus ATCC 9537 D15063
Rhodobacter capsulatus ATCC 11166 D16428
Rhodobacter rubrum ATCC 11770 D30778
Beijerinckia indica ATCC 9039 M59066
Caulobacter sp. MCS 6 M38811
Hyphomonas sp. MHS 3 M38812
Hyphomicrobiun vulgare MC-750 X35182
Roseobacter litoralis ATCC 49556 X78512
Azospirillum lipapha ATCC 29708 X79729
Azospirillum trakeze ATCC 11770 X79737
Azospirillum brasilense Sp 7 X79739
Azospirillum amazonesense Y2 X79742
Xanthobacter flavus JW/KR-E1 X94206
Pedomonimucum manganicum ACM 3038 X79691

* Substrain MB 58.
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 *N₂*-fixing isolates are shown in Table 3. The genetic relationships among the *N₂*-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 *N₂*-fixing coffee iso-

**TABLE 3. Origins of representative *N₂*-fixing bacteria recovered from the coffee environment**

<table>
<thead>
<tr>
<th>MLEE division (ET)</th>
<th>Type of isolate</th>
<th>Strain designation</th>
<th>Isolate recovered from:</th>
<th>Plant age</th>
<th>Cultivar</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (1)</td>
<td><em>A. diazotrophicus</em></td>
<td>CFN-Cf13</td>
<td>Stem tissue</td>
<td>2 mo</td>
<td>Catuai</td>
<td>Xicotepec, Puebla</td>
</tr>
<tr>
<td>I (11)</td>
<td><em>A. diazotrophicus</em></td>
<td>CFN-Cf50</td>
<td>Root tissue</td>
<td>6 mo</td>
<td>Catuai</td>
<td>Xicotepec, Puebla</td>
</tr>
<tr>
<td>I (9)</td>
<td><em>A. diazotrophicus</em></td>
<td>CFN-Cf52</td>
<td>Root tissue</td>
<td>1 yr</td>
<td>Catarra</td>
<td>Atoyac, Guerrero</td>
</tr>
<tr>
<td>I (13)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf52</td>
<td>Root tissue</td>
<td>1 yr</td>
<td>Catarra</td>
<td>Atoyac, Guerrero</td>
</tr>
<tr>
<td>I (6)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf01</td>
<td>Rhizosphere</td>
<td>5 yr</td>
<td>Garnica</td>
<td>Huiztizlan, Puebla</td>
</tr>
<tr>
<td>I (8)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf05</td>
<td>Root tissue</td>
<td>5 yr</td>
<td>Garnica</td>
<td>Huiztizlan, Puebla</td>
</tr>
<tr>
<td>I (12)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf51</td>
<td>Rhizosphere</td>
<td>5 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>I (14)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf53</td>
<td>Rhizosphere</td>
<td>3 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>I (10)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf58</td>
<td>Rhizosphere</td>
<td>5 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>I (15)</td>
<td>NAP</td>
<td>CFN-Cf56</td>
<td>Rhizosphere</td>
<td>3 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>III (15)</td>
<td>APL</td>
<td>UAP-Cf59</td>
<td>Rhizosphere</td>
<td>5 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>III (16)</td>
<td>APL</td>
<td>CFN-Cf60</td>
<td>Rhizosphere</td>
<td>3 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>IV (17)</td>
<td>DOR</td>
<td>CFN-Cf55</td>
<td>Rhizosphere</td>
<td>3 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>IV (18)</td>
<td>DOR</td>
<td>UAP-Cf57</td>
<td>Rhizosphere</td>
<td>5 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
<tr>
<td>V (19)</td>
<td>SAd</td>
<td>CFN-Cf54</td>
<td>Rhizosphere</td>
<td>5 mo</td>
<td>Catarra</td>
<td>Tapachula, Chiapas</td>
</tr>
</tbody>
</table>

* 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.
* Coffee plants growing in a nursery.
* Coffee plants growing under field conditions.
* NAP, non-acid-producing isolate.
* APL, acid-producing liquid isolate.
* DOR, dark-orange isolate.
* SAd, isolate with colonial features similar to those of *A. diazotrophicus.*
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 CO₂ and H₂O (Table 4), phenotypic features which are considered (8, 29) fundamental for the identification of the genus *Acetobacter*.

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

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**FIG. 2.** Genetic relationships of ETs identified among *A. diazotrophicus* isolates recovered from well-known hosts and N₂-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.

**TABLE 4.** Phenotypic characteristics of N₂-fixing acetic acid bacteria isolated from the coffee plant environment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Oxidase</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Catalase</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Oxidation of ethanol to acetic acid</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Oxidation of acetic acid to CO₂ and H₂O</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Water-soluble brown pigments on GYC</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dark-orange colonies on LGI plates</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Brownish colonies on potato agar with 10% sugar</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Growth with 30% D-glucose</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Growth with 30% sucrose</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Yellow surface pellicle formation and pH below 3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C₂H₂ reduction activity (N₂ fixation)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Phenotypic characteristics were positive (+) for each strain.*

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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 EcoRI 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). N2-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-

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**TABLE 5. Genetic characteristics of some N₂-fixing acetobacters recovered from the coffee plant environment**

<table>
<thead>
<tr>
<th>MLEE division (ET)</th>
<th>Type of isolate</th>
<th>Reference strain</th>
<th>sizes (kb)</th>
<th>DNA-DNA homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (3)</td>
<td><em>A. diazotrophicus</em></td>
<td>PAI 5ₜ</td>
<td>9.0, 2.0, 1.25 9.3, 3.6, 2.3, 1.6</td>
<td>100.0</td>
</tr>
<tr>
<td>I (11)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf 05</td>
<td>9.0, 2.0, 1.25 9.3, 3.6, 2.3, 1.6</td>
<td>104.0</td>
</tr>
<tr>
<td>I (8)</td>
<td><em>A. diazotrophicus</em></td>
<td>CFN-Cf 52</td>
<td>9.0, 2.0, 1.25 9.3, 3.6, 2.3, 1.6</td>
<td>80.0</td>
</tr>
<tr>
<td>I (9)</td>
<td><em>A. diazotrophicus</em></td>
<td>UAP-Cf 58</td>
<td>9.0, 2.0, 1.25 9.3, 3.6, 2.3, 1.6</td>
<td>96.0</td>
</tr>
<tr>
<td>I (12)</td>
<td><em>A. diazotrophicus</em></td>
<td>CFN-Cf 50</td>
<td>9.0, 2.0, 1.25 9.3, 3.6, 2.3, 1.6</td>
<td>72.0</td>
</tr>
<tr>
<td>I (13)</td>
<td><em>A. diazotrophicus</em></td>
<td>CFN-Cf 56</td>
<td>7.6, 3.5, 1.20, 1.0 9.3, 3.6, 2.3, 1.6</td>
<td>77.0</td>
</tr>
<tr>
<td>III (15)</td>
<td>APL</td>
<td>UAP-Cf 59</td>
<td>Not detected 9.3, 3.6, 1.6</td>
<td>12.0</td>
</tr>
<tr>
<td>III (16)</td>
<td>APL</td>
<td>CFN-Cf 60</td>
<td>Not detected 9.3, 3.6, 1.6</td>
<td>15.0</td>
</tr>
<tr>
<td>IV (17)</td>
<td>DOR</td>
<td>CFN-Cf 55</td>
<td>9.0, 2.0, 1.20 9.3, 3.6, 2.3, 1.6</td>
<td>14.0</td>
</tr>
<tr>
<td>IV (18)</td>
<td>DOR</td>
<td>UAP-Cf 57</td>
<td>9.0, 2.0, 1.20 9.3, 3.6, 2.3, 1.6</td>
<td>15.0</td>
</tr>
<tr>
<td>V (19)</td>
<td>SAd</td>
<td>CFN-Cf 54</td>
<td>6.6, 2.1, 1.15 9.3, 3.6, 2.3, 1.6</td>
<td>11.0</td>
</tr>
</tbody>
</table>

*Divisions and ETs were based on MLEE assays.
Types described in Table 3, footnotes d, e, f, and g.
Bands from total EcoRI DNA fingerprints hybridized as described in Materials and Methods.
Homology to the control strain PAI 5ₜ.
* A. diazotrophicus strains recovered from sugarcane used as controls.
ND, not determined.
the coffee plant environment, digested with digestion enzyme. Genomic DNA from the strains recovered from Table 5. The six strains of N2-fixing acetobacters correspond to the expected 1.24-kb band (26) (data not shown).

Proteobacteria lack the oxydans ever, all Acidiphilium rDNA are characterized by two Gluconobacter I conserved sites.

A. diazotrophicus M59066) lacks one of the Acidiphilium sites in their SSU rDNA, except for A. diazotrophicus Sph I site at base 485 as deduced from the reported sequence PAl 5T.

homology levels, ranging from 11 to 15% with reference strain isolates (MLEE division III) and DOR acetobacters from division I (except strain CFN-Cf 56) analyzed were expected to observe one hybridizing band of 450 bp with the expected to observe one hybridizing band of 450 bp with the probe used when the DNA was digested with SphI. However, only one SSU rRNA hybridizing band of 1.3 kb was observed in A. diazotrophicus PAI 5T 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 SphI site. If such were the case, then the Acetobacteraceae and Acidiphilum spp. would have only two SphI conserved sites. Gluconobacter and Acetobacter SSU rDNA are characterized by two NcoI restriction sites. However, all Acidiphilum and Rhodopila species and Gluconobacter oxydans lack the NcoI restriction site at the base corresponding to nucleotide 110 of A. diazotrophicus. The rest of the a-Proteobacteria analyzed lack at least one site for each restriction enzyme. Genomic DNA from the strains recovered from the coffee plant environment, digested with NcoI 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 N2-fixing acetobacters corresponding to division I (except strain CFN-Cf 56) analyzed were related to A. diazotrophicus PAI 5T 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 PAI 5T. 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 PAI 5T.

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 N2 (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 NcoI and SphI (data not shown).

Moreover, we have considered it suitable to assign these N2-fixing isolates to the genus Acetobacter because they were capable of oxidizing ethanol, first to acetic acid and then further to CO2 and H2O (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 N2-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 Fraturelia (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 N2 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 N2-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.

FIG. 5. Diagrammatic representation of distinctive restriction sites SphI and NcoI 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 PAI 5T sequence but not detected after Southern hybridization; c, site present in Azospirillum ipoforum 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.

600 bp SSU rDNA probe

Acetobacter spp., Gluconobacter spp. and Acidomonas methanolica

Acidiphilum spp.

Rhodopila globiformis
different α-Proteobacteria
Furthermore, these *Acetobacter* isolates differed in SSU rRNA RFLP patterns, and they had a very low level of DNA homology with *A. diazotrophicus* PAI 5T. These data are strong evidence to designate other diazotrophic species of the genus *Acetobacter*, but more N2-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 PAI 5T.

Natural habitats of acetic acid bacteria are sugar and alcohol solutions, with flowers and many fruits being excellent habitats and strain PAI 5T. Low DNA-DNA homology values between strain CFN-Cf 56. However, 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 PAI 5T.

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 (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 N2-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 frequency 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 N2-fixing acetobacters from coffee plant fruit or mealybugs (*Planococcus citrini*). 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 more N2-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 N2-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.

**ACKNOWLEDGMENTS**

We are grateful to Michael Hynes and Michael Dunn for reviewing the paper and to R. Bustillos-Cristales, Minerva Rosas, L. Martínez-Aguilar, and M. E. Nava-Herrera for technical assistance. We thank C. Abarca-Ocampo (SAGAR-Guerrero) for his support in collecting coffee plants and the associated mealybugs.

This work was supported in part by grant UNAM-DGAPA-IN209496.

**REFERENCES**


