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Limited Genetic Diversity in the Endophytic Sugarcane Bacterium *Acetobacter diazotrophicus*

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Acetobacter diazotrophicus **isolates that originated from different sugarcane cultivars growing in diverse geographic regions of Mexico and Brazil were shown to have limited genetic diversity. Measurements of polymorphism in the electrophoretic mobilities of metabolic enzymes revealed that the mean genetic diversity per enzyme locus (among the four electrophoretic types distinguished) was 0.064. The results of the genetic analysis indicate that the genetic structure of *A. diazotrophicus* is clonal, with one largely predominant clone. Plasmids were present in 20 of 24 isolates, and the molecular sizes of the plasmids ranged from 2.0 to 170 kb. Two plasmids (a 20- to 24-kb plasmid detected in all 20 plasmid-containing isolates and a 170-kb plasmid observed in 14 isolates) were highly conserved among the isolates examined. Regardless of the presence of plasmids, all of the isolates shared a common pattern of *nif* structural gene organization on the chromosome.**

Considerable variability exists within individual soil bacterial species, including members of the genera *Azospirillum* (10, 28), *Bacillus* (21), *Bradyrhizobium* (33, 41), and *Rhizobium* (12, 27). An analysis of the genetic structures of populations, as estimated by allele frequencies and multilocus genotypes, revealed high levels of genetic diversity in *Rhizobium leguminosarum* biovar phaseoli (27) and in the soil bacterium *Pseudomonas cepacia*. In *Pseudomonas cepacia* the diversity was correlated with soil environment variability (22). The levels of genetic diversity in species of bacteria may be related to habitat. Less variability has been observed in *Escherichia coli* (38), *Haemophilus influenzae* (23), *Yersinia ruckeri* (32), and *Pasteurella piscicida* (18), bacteria that predominantly or exclusively live under constant conditions inside organisms.

Over the last few years there has been great interest in plant-associated bacteria. Research to improve crop responses has emphasized the study of nitrogen-fixing bacteria indigenous to the rhizosphere, but little is known about nonrhizosphere nitrogen-fixing bacterial populations associated with plants. Recently, *Acetobacter diazotrophicus*, an N_2-fixing bacterium, has been isolated from sugarcane roots and stems (5, 13, 16). This species has also been recovered from both *Pennisetum purpureum* cv. Cameroon and sweet potato (8), as well as from different genera of mealybugs associated with sugarcane plants (1). In this paper we report that there is limited genetic diversity among isolates of *A. diazotrophicus* recovered from sugarcane plants growing in diverse geographic regions of Mexico and Brazil, and we also show that this species harbors highly conserved small and large plasmids.

MATERIALS AND METHODS

Bacterial strains. The strains of *A. diazotrophicus* which we used were isolated from the inside tissues of stems or roots of sugarcane plants cultivated in diverse geographic regions of Mexico (13). Only isolates recovered from different plants were considered to be different. These strains were maintained in 10% glycerol at −70°C. Brazilian strains PAl 5^T (= ATCC 49037^T) (T = type strain), PPe 4 (= ATCC 49038), and PR 2 (= ATCC 49039) were kindly supplied by J. Döbereiner. *E. coli* HB101 (11) and GO 102 (9), the latter kindly supplied by R. B. Gennis, and *Rhizobium etli* CFN 42 (34) were used as described below.

Media and cultural conditions. SYP medium (1.0% sucrose, 0.1% yeast extract, 0.1% K_2HPO_4, 0.3% KH_2PO_4; pH 6.2) was used to grow *A. diazotrophicus* isolates. *E. coli* strains were grown in Luria broth. *R. etli* CFN 42 was grown in peptone-yeast extract liquid medium (25). The incubation temperature used was 29°C, and the cultures were shaken at 200 rpm.

Multilocus enzyme electrophoresis. Cultures grown for 36 h in 40 ml of SYP medium were harvested by centrifugation at 12,300 × g for 10 min at 4°C, and the resulting preparations were suspended in 0.3 ml of 10 mM $MgSO_4 \cdot 7H_2O$ containing 300 μg of lysozyme and incubated for 10 min at room temperature. Each suspension was frozen at −70°C for 15 min and defrosted; then the process was repeated. Lysates were stored at −70°C.

The procedures used for starch gel electrophoresis and selective staining of enzymes have been described previously by Selander et al. (37). The following 11 metabolic enzyme activities were assayed: alcohol dehydrogenase, malate dehydrogenase, xanthine dehydrogenase, lysine dehydrogenase, leucine dehydrogenase, isocitrate dehydrogenase, indophenol oxidase (superoxide dismutase), glucose-6-phosphate dehydrogenase, phosphoglucomutase, hexokinase, and esterases. The buffer system used was Tris-citrate (pH 8.0). The electrophoretic mobility of each enzyme was determined three times. Distinctive combinations of alleles for the 11 enzyme loci (multilocus genotypes) were designated different electrophoretic types (ETs) (37). The ET was determined for each isolate. The level of genetic diversity for each enzyme locus was calculated as described previously (37).

Total-DNA isolation and DNA restriction. Total DNA was isolated as described previously (2). DNA was digested with restriction endonuclease *Eco*RI. Total-DNA restriction fragments were electrophoresed in vertical 1.0% agarose gels in Tris-acetate buffer (40 mM Tris-acetate, 2 mM EDTA; pH 8) at 40 V for 13 h at 4°C. The gels were stained with ethidium bromide and photographed under UV illumination.

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TABLE 1. Genetic diversity at 11 enzyme loci

Enzyme locus[a]	No. of alleles	Genetic diversity[b]
ADH	1	0.000
MDH	1	0.000
XDH	1	0.000
LYD	1	0.000
LED	1	0.000
IDH	2	0.106
IPO	1	0.000
G6P	2	0.203
PGM	1	0.000
HEX	2	0.106
EST	2	0.291
Mean	1.36	0.064

[a] ADH, alcohol dehydrogenase; MDH, malate dehydrogenase; XDH, xanthine dehydrogenase; LYD, lysine dehydrogenase; LED, leucine dehydrogenase; IDH, isocitrate dehydrogenase; IPO, indophenol oxidase; G6P, glucose-6-phosphate dehydrogenase; PGM, phosphoglucomutase; HEX, hexokinase; EST, esterases.

[b] $h = 1 - \Sigma x_i^2[n/(n-1)]$, where h is the genetic diversity, x_i is the frequency of the ith allele, and n is the number of ETs.

Plasmid content and electrophoresis procedure. Plasmid profiles were analyzed by the procedure of Hirsch et al. (15). Plasmid DNAs were electrophoresed in vertical 0.75% agarose gels prepared in Tris-borate buffer (45 mM Tris-borate, 2 mM EDTA; pH 8) at 120 V for 13 h. In addition, small plasmids were also identified by the alkaline lysis method (31). Small plasmids were electrophoresed for 4 h at 120 V in vertical 1.0% agarose gels prepared in Tris-acetate buffer. Electrophoresis was performed at 4°C, and bands were visualized as described above. Plasmid molecular weights were estimated by using *R. etli* CFN 42 and *E. coli* HB101(pRK2013), GO 102(pFH101), and S17(pSUP202 carrying a 13-kb insert) as reference markers.

Plasmid and DNA hybridization. Total-DNA digests or plasmids were transferred from gels to nylon filters by the Southern procedure (40). Individual *A. diazotrophicus* plasmids from Brazilian strain PR 2 were purified from agarose gels with Geneclean (Bio 101, Inc., La Jolla, Calif.) and used as probes. To localize the *nif* genes of *A. diazotrophicus*, filters were hybridized with pCQ12, which contains a 4.1-kb segment of the *nifHDK* region of *R. etli* CFN 42 (29). ^{32}P-labelled probes were prepared by nick translation (31), and the DNA-DNA hybridization procedure was performed as described previously (20).

RESULTS

Multilocus enzyme electrophoresis. Of the 11 metabolic enzymes assayed, 4 were polymorphic with only two alleles per locus (Table 1). Four distinctive ETs were identified among the 24 *A. diazotrophicus* isolates studied (Table 2), and the mean level of genetic diversity for the 11 enzyme loci was found to be 0.064 (Table 1). According to Selander et al. (35, 36), ETs mark clones; if a particular clone differs from the most frequent clone at a single gene locus, then it is called a subclone. We observed only four ETs. ET 1 represented 18 of the 24 isolates (75%), including Brazilian isolate PR 2. ET 1 and ET 4 represented two clones, while ET 2 and ET 3 were considered two subclones of the predominant clone, clone ET 1 (Table 1). Clones ET 1 and ET 4 differed in alleles at two enzyme gene loci (isocitrate dehydrogenase and hexokinase). Subclones ET 2 and ET 3 differed from ET 1 at the esterase and glucose-6-phosphate dehydrogenase enzyme loci, respectively.

The genetic relationships among the four ETs are illustrated by a dendogram in Fig. 1. Predominant clone ET 1 is widely distributed; it was recovered from 11 different sugarcane

TABLE 2. Origins of *A. diazotrophicus* strains, ETs, DNA patterns, and numbers and sizes of plasmids

Sugarcane cultivar	Strain	ET	DNA pattern	No. of plasmids	Plasmid size(s) (kb)
—[a]	PR 2	1	I	2	170, 24
MEX 57 473	UAP 5701	1	I	2	170, 24
MEX 57 473	UAP 5702	1	I	2	170, 24
CP 72 2086	UAP 7210	1	I	2	170, 24
MEX 69 290	UAP 6925	1	I	2	170, 24
MEX 69 290	UAP 6926	1	I	2	170, 24
Cristal	UAP 0030	1	I	2	170, 24
MEX 79 546	UAP 7936	1	I	2	170, 24
Z MEX 55 32	UAP 5560	1	I	2	170, 24
MEX 52 17	UAP 5275	1	I	2	170, 24
MEX 73 523	UAP 7305	1	ND[b]	2	170, 22
MEX 73 523	UAP 7306	1	ND	2	170, 22
MEX 73 523	UAP 7308	1	ND	2	170, 22
MEX 73 523	UAP 7309	1	ND	2	170, 22
MEX 56 476	UAP 5665	1	II	3	93, 22, 2.2
MEX 80 499	UAP 8070	1	II	3	93, 22, 2.2
MEX 73 523	UAP 7307	1	III	2	20, 5.5
Cristal	UAP 0020	2	IV	1	20
Cristal	UAP 0021	2	IV	1	20
MY 55 14	UAP 5540	2	IV	1	20
—	PAI 5^T	3	V	NV[c]	
Cristal	UAP 0050	3	VI	NV	
—	PPe 4	4	VI	NV	
MY 55 14	UAP 5541	1	VI	NV	

[a] —, for cultivar information see reference 14.

[b] ND, not determined.

[c] NV, none visualized.

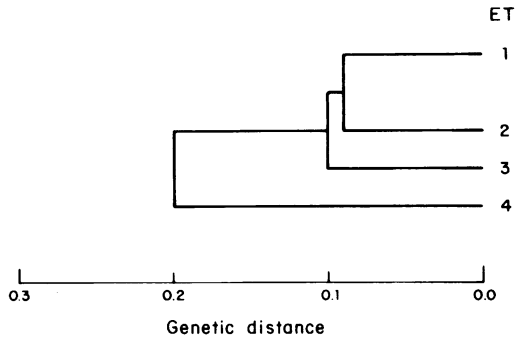

FIG. 1. Genetic relationships of four ETs identified among 24 *A. diazotrophicus* isolates recovered from sugarcane plants.

varieties cultivated in Mexico in diverse geographic regions up to 500 km apart (13). These sugarcane varieties were introduced commercially into Mexico between 1952 and 1980, as indicated by the first two numbers of each variety designation (Table 1). Brazilian isolate PR 2, which was assigned to the ET 1, was collected from sugarcane cultivated 9,000 km from Mexico (5). Subclone ET 3, represented by type strain PAl 5 of *A. diazotrophicus* and one Mexican isolate, was recovered from sugarcane plants from widely separated geographic regions. ET 4 was represented by only a single isolate (PPe4).

DNA fingerprints. An analysis of all of the *Eco*RI DNA fingerprints revealed that the patterns differed mainly in fragments ranging in size from 6.5 to 23.1 kb. Otherwise, the patterns of all of the isolates were almost identical for small fragments (less than 6.5 kb long) (data not shown). The fingerprints obtained represented six patterns (Table 2). The majority of the isolates produced one pattern (DNA pattern I). Two other DNA patterns (patterns III and V) were each found in only a single isolate.

Plasmid patterns. Four different plasmid profiles were found, corresponding generally to ET 1 and ET 2. Most of the Mexican isolates and one Brazilian isolate contained at least two plasmids (Table 2). Fourteen isolates harbored two common, highly conserved plasmids, as revealed by agarose gel electrophoresis and hybridization assays, a large 170-kb plasmid (pAd170) and a smaller 20- to 24-kb plasmid (pAd24) (Fig. 2). Isolates UAP 5665 and UAP 8070, which were obtained from different locations and sugarcane cultivars, contained a 93-kb plasmid (pAd93) and did not contain pAd170. Plasmid pAd93 was shown to be homologous to pAd170 by hybridization assays (Fig. 2). These two isolates also harbored a small (ca. 2.0-kb) plasmid in addition to pAd24 (Fig. 3). Four isolates contained only pAd24, and one of these isolates also contained a 5.5-kb plasmid (pAd5). Four isolates, including type strain PAl 5 of *A. diazotrophicus*, did not contain any plasmids (Fig. 2). The absence of pAd24, pAd93, and pAd170 was corroborated by the lack of hybridization of DNA fingerprints when we used total DNAs from plasmidless isolates digested with *Eco*RI and hybridized with purified 24- and 170-kb plasmids from Brazilian strain PR 2 (data not shown).

***nifHDK* patterns.** Total *Eco*RI DNA digests from *A. diazotrophicus* isolates were hybridized to *R. etli nifHDK* genes. Three common hybridizing bands (9.0, 2.0, and 1.25 kb) were found in all of the isolates examined (Fig. 4). Blots of Hirsch type gels were also hybridized to *nifHDK* genes, and the lack of hybridization signals corresponding to the plasmids was interpreted to indicate that nitrogen fixation genes were located on the chromosome (data not shown).

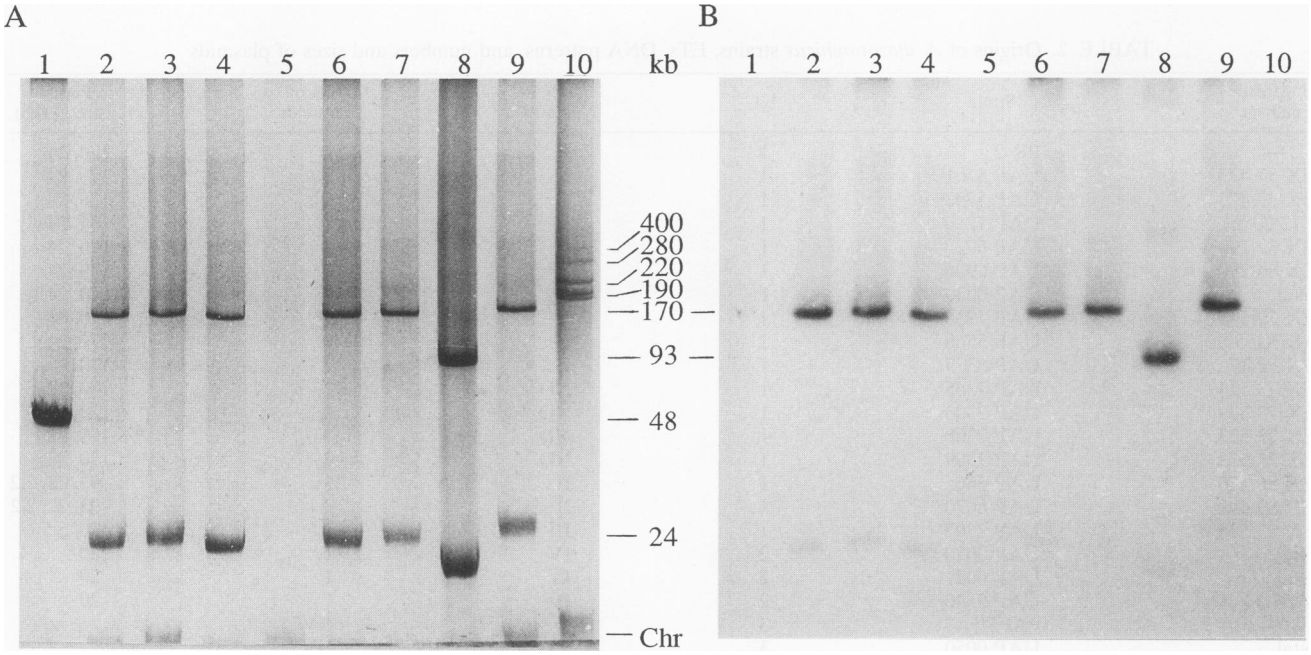

FIG. 2. (A) Agarose gel electrophoresis of plasmids obtained by the Hirsch procedure. (B) Autoradiogram of a Southern blot of the plasmid profile after hybridization with plasmid pAd170 from Brazilian isolate PR 2. Lanes 1, *E. coli* HB101 harboring pRK2013 (48 kb), used as a marker; lanes 2 through 9, *A. diazotrophicus* UAP 5701, UAP 6925, UAP 7936, PAl 5^T, PR 2, UAP 5560, UAP 5665, and UAP 5275, respectively; lanes 10, *R. etli* CFN 42 harboring plasmids a and b (190 kb), plasmid c (220 kb), plasmid d (*sym* plasmid, 280 kb), and plasmid e (400 kb), used as a marker. Chr, chromosome.

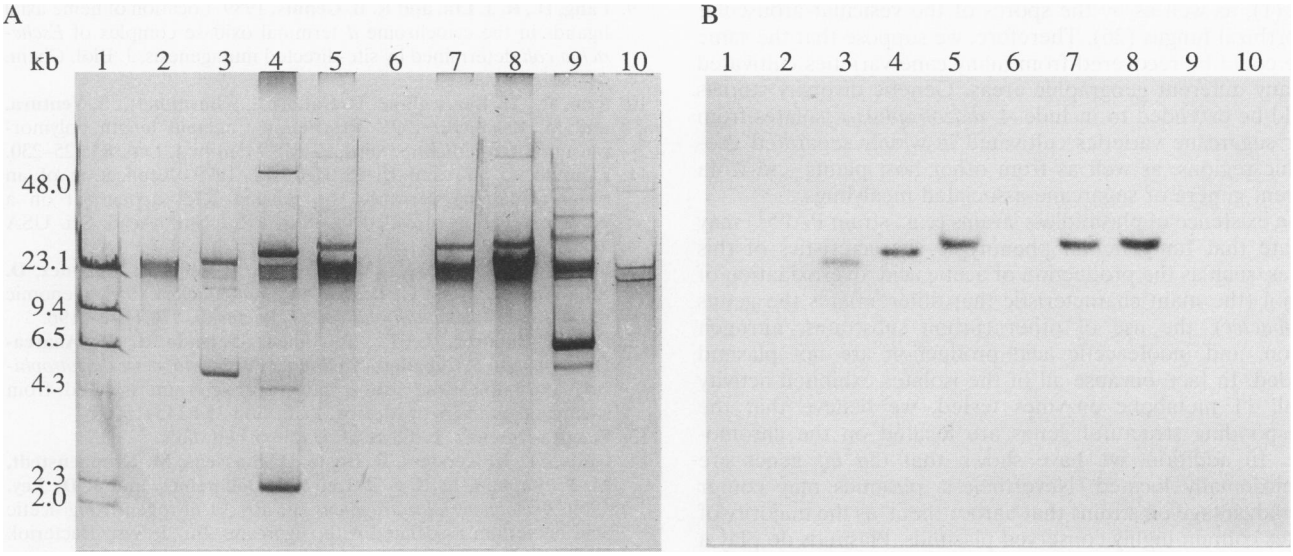

FIG. 3. (A) Agarose gel electrophoresis of plasmids obtained by the alkaline lysis method. (B) Autoradiogram of a Southern blot of the plasmid profile after hybridization with plasmid pAd24 from Brazilian isolate PR 2. Lanes: 1, lambda DNA digested with *Hin*dIII; lanes 2 through 8, *A. diazotrophicus* PAl 5^T, UAP 7307, UAP 5665, PR 2, PPe4, UAP 5560, and UAP 7210, respectively; lanes 9, *E. coli* GO 102 harboring pFH101 (7.7 kb); lanes 10, *E. coli* HB101 and S17 harboring plasmids pRK 2013 (48 kb) and pC131 (17 kb), respectively, used as markers.

DISCUSSION

In this paper we describe an analysis of the ETs, the DNA fingerprint patterns, and the plasmid profiles of different *A. diazotrophicus* strains. The genetic diversity among the *A. diazotrophicus* isolates which we studied was limited. The results of a multilocus enzyme electrophoresis analysis performed to assess variation in 11 metabolic enzyme loci indicated that these isolates are homogeneous in their chromosomal structural genes and revealed levels of genetic diversity that are among the lowest levels that have been reported for bacterial species. The limited genetic variability among isolates which we observed was consistent with the conserved *nifHDK* gene organization patterns. In addition, Gillis et al. (14) found that the gel electrophoretic cellular protein patterns of seven *A. diazotrophicus* strains are very similar, indicating that these isolates are closely related to one another. Moreover, membrane protein electropherograms revealed similar findings (13a).

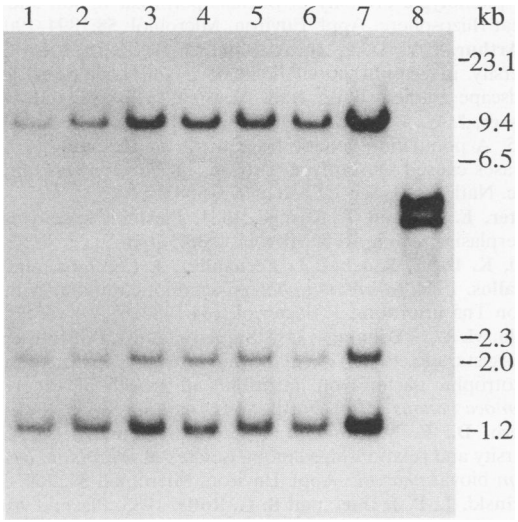

FIG. 4. Autoradiogram of a Southern blot of the total *Eco*RI DNA fingerprints hybridized with the *nifHDK* probe of *R. etli* CFN 42. Lanes 1 through 7, *A. diazotrophicus* UAP 8070, PAl 5^T, PR 2, UAP 5560, PPe4, UAP 5665, and UAP 5541, respectively; lane 8, *R. etli* CFN 42, used as a control.

Selander et al. (39) have pointed out that "isolates of identical ET are considered members of the same clone, because evolutionary convergence to the same multilocus genotype is highly improbable." Despite the low number of isolates analyzed, the results obtained in this study suggest that *A. diazotrophicus* has a clonal genetic structure on the basis of the wide distribution of one genotype. This type of genetic structure has also been observed for other bacteria (23, 32, 35, 39).

It has been suggested that ecological factors and host specialization contribute to the species diversity of the genus *Legionella* (39). Also, it has been shown that genetic diversity among isolates of *Pseudomonas cepacia* is related to environmental variability in soils (22). Considering these data, we think that the limited genetic diversity of the modern commercial sugarcane varieties (17) and the relatively constant environment inside sugarcane stems, the habitat of *A. diazotrophicus*, may explain the limited genetic diversity of this bacterium.

Dispersal of *A. diazotrophicus* by sugarcane seeds may not occur as we have not been able to isolate the bacteria from seeds. Long-distance dispersal of *A. diazotrophicus* may be explained by considering two characteristics of cultivated sugarcane: (i) sugarcane is normally propagated vegetatively from stem cuttings, where *A. diazotrophicus* occurs endophytically (4, 13); and (ii) frequently, many commercial varieties from one country are cultivated in another country (for instance, cultivars CP 72 2086 and My 55 14 from the United States and Cuba, respectively [Table 2], and other cultivars from different countries, such as Brazil and Australia, are cultivated in Mexico). It seems probable that *A. diazotrophicus* is spread among cane cultivars by the mealybugs associated with sugar-

cane (1), as well as by the spores of the vesicular-arbuscular mycorrhizal fungus (26). Therefore, we suppose that the same clone could be recovered from many cane varieties cultivated in many different geographic areas. Genetic diversity studies should be extended to include *A. diazotrophicus* isolates from other sugarcane varieties cultivated in widely separated geographic regions, as well as from other host plants and from different genera of sugarcane-associated mealybugs.

The existence of plasmidless strains (e.g., strain PAl 5^T) may indicate that fundamental phenotypic characteristics of this species, such as the production of acetic acid, overoxidation of ethanol (the main characteristic that differentiates the genus *Acetobacter*), the use of other carbon substrates, nitrogen fixation, and indoleacetic acid production are not plasmid encoded. In fact, because all of the isolates exhibited activity for all 11 metabolic enzymes tested, we believe that the corresponding structural genes are located on the chromosome. In addition, we have shown that the *nif* genes are chromosomally located. Nevertheless, plasmids may confer some advantage on strains that harbor them, as the majority of isolates contain highly conserved plasmids. Plasmids do play a role in other bacterium-plant or bacterium-insect interactions (3, 6, 7, 19, 24, 30), and we speculate that the *A. diazotrophicus* plasmids could contribute to the fitness of *A. diazotrophicus*-sugarcane or *A. diazotrophicus*-mealybug associations.

To test the hypothesis that the degree of genetic diversity within a bacterial species is related to habitat, it would be interesting to determine if a limited genetic diversity as we have described for *A. diazotrophicus* is a general characteristic of endophytic bacterial populations.

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