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Genetic Structure of *Acetobacter diazotrophicus* Populations and Identification of a New Genetically Distant Group

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A total of 55 isolates of *Acetobacter diazotrophicus* recovered from diverse sucrose-rich host plants and from mealybugs associated with sugarcane plants were characterized by the electrophoretic mobilities of 12 metabolic enzymes. We identified seven different electrophoretic types (ETs), six of which are closely related within a genetic distance of 0.195 and exhibit high DNA-DNA homology. The seventh ET was largely divergent, separated at a genetic distance of 0.53, and had only 54% DNA homology to the reference strain. Strains corresponding to ET 7 could represent a distinct nitrogen-fixing species of the genus *Acetobacter*. More genetic diversity was found in isolates from Brazil than in those from Mexico, probably due to the very different crop nitrogen fertilization levels used.

Cane sugar is produced commercially in over 70 countries around the world (31). It is an important agricultural product which is used for domestic consumption and export. More than 150 by-products may be obtained from sugarcane (33). For instance, ethanol obtained by fermentation and distillation of sugarcane juice provides fuel for 4 million motor vehicles in Brazil, and 7 million other vehicles use gasohol containing 10 to 22% ethanol (5).

Commonly, very high levels of nitrogen fertilizers (120 to 300 kg of N per ha) are used in sugarcane crops in countries such as Mexico, Venezuela, Cuba, and the United States (Hawaii). In contrast, sugarcane crops in Brazil do not receive more than 50 kg of nitrogen fertilizer (37), and neither cane yields nor soil N reserves appear to diminish after decades of culture (5). Recent experiments estimated that the contribution of biological nitrogen fixation to the sugarcane cultivars ranged from 50 to 80% of total plant nitrogen (5, 52).

Nitrogen-fixing bacterial species, such as *Enterobacter cloacae*, *Bacillus polymyxa*, *Klebsiella pneumoniae*, *Azotobacter vinelandii*, and *Azospirillum* spp., are commonly isolated from different internal or external parts of sugarcane plants (37; unpublished results). Recently, other diazotrophs (*Herbaspirillum seropedicae* [3] and *Acetobacter diazotrophicus* [11, 17]) have been isolated from inside tissues of roots and stems of sugarcane. At present, which of these bacteria are the most important in plant-associated biological nitrogen fixation remains unknown. However, *A. diazotrophicus* has been suggested as a strong candidate responsible for the N_2 fixation observed in field experiments with sugarcane (5, 51).

A. diazotrophicus has also been recovered from other sucrose-rich host plants such as sweet potato (*Ipomoea batatas*) and Cameroon grass (*Pennisetum purpureum*), which are vegetatively propagated (15), as well as from different genera of mealybugs associated with sugarcane plants (1).

Multilocus enzyme electrophoresis (MLEE) has been used extensively to measure genotypic diversity and genetic structure of natural populations of many bacterial species (43). Such studies have revealed that the levels of genetic variability differ greatly among species. For instance, *Yersinia ruckeri* organisms exhibit a genetic diversity as low as 0.014 (38), while oral streptococci show a diversity as high as 0.857 (19). Between these extremes are found very different pathogenic bacterial species of plants (14), animals (4, 30), and humans (10, 13, 29, 42), as well as soil bacterial species, including *Bacillus* spp. (9, 21), a *Bradyrhizobium* sp. (6), *Pseudomonas cepacia* (27), and *Rhizobium* spp. (16, 24, 35). Genetic diversity levels have mainly been related to effective population size (28, 45) and recent evolutionary origin of the species (12, 28), along with ecological factors (27, 45) and niche specialization (14, 23, 30, 40).

Taking into account that "the characterization and understanding of natural populations of useful bacteria may save work and money in the development of low-risk, successful biotechnology" (46), we considered it of interest to extend our previous studies on genetic diversity of *A. diazotrophicus* isolated from sugarcane (8) to include bacteria isolated from other host plants such as sweet potato and *P. purpureum* and from the mealybug *Saccharicoccus sacchari*. In this work, we report the genetic relatedness among isolates recovered mainly from Mexico and Brazil. We show evidence of a new genetically distinct group.

MATERIALS AND METHODS

Isolation. *A. diazotrophicus* strains were isolated from the inside tissues of stems or roots of sugarcane plants cultivated in Mexico, as described previously (17).

Each mealybug colony, identified as *S. sacchari*, sampled from stems of independent sugarcane plants was rinsed with 0.01% (vol/vol) Tween 40 in 10 mM $MgSO_4 \cdot 7H_2O$ until the liquid was clear. Subsequently, mealybugs were immersed in 1% chloramine T for 5 min and then washed three times in 10 mM $MgSO_4 \cdot 7H_2O$. Insects were macerated in 1.0 ml of sterile distilled water, and aliquots were inoculated into media for isolation of *A. diazotrophicus*, as described previously (17).

Mealybug colonies and sugarcane varieties sampled in Mexico were from diverse cane-growing areas up to 1,500 km apart; cane-growing areas of Brazil were located up to 2,500 km apart.

Bacterial strains. Strains and their sources are shown in Table 1. Most of the strains were recovered from hosts collected in Mexico and Brazil, but the samples also included two isolates from Australia and one from Uruguay. Strain 7.10RM was recovered from within spores of the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* obtained from sweet potatoes grown in soil inoculated with a mixture of the fungus and strain PAl 5^T of *A. diazotrophicus*. Only isolates

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TABLE 1. ETs, host species, and locality for 55 isolates of *A. diazotrophicus*

ET	Strain	Host species	Locality	Source
1	CFNE 501	Stem, Z Mex 55 32[a]	Veracruz, Mexico	This work
1	CFNE 502	Stem, Mex 69 290[a]	Veracruz, Mexico	This work
1	CFNE 503	Stem, RD 75 01[b]	Veracruz, Mexico	This work
1	CFNE 504	Stem, Mex 73 523[a]	Veracruz, Mexico	This work
1	CFNE 505	Stem, Mex 68 P23[a]	Sinaloa, Mexico	This work
1	CFNE 506	Stem, RD 75 11[b]	Sinaloa, Mexico	This work
1	CFNE 507	Stem, RB 73 9953[b]	Sinaloa, Mexico	This work
1	CFNE 508	Stem, SP 70 1005[b]	Sinaloa, Mexico	This work
1	CFNE 509	Stem, SP 70 3370[b]	Sinaloa, Mexico	This work
1	CFNE 510	Stem, RB 72 1012[b]	Sinaloa, Mexico	This work
1	CFNE 513	Stem, My 55 14[b]	Puebla, Mexico	This work
1	CFNE 515	Stem, SP 70 1248[b]	Puebla, Mexico	This work
1	CFNE 516	Roots, SP 70 1248[b]	Puebla, Mexico	This work
1	CFNE 521	Roots, CP 72 2086[b]	Veracruz, Mexico	This work
5	PAl 3	Roots, sugarcane	Alagoas, Brazil	CNPAB collection[c]
1	PRJ 6	Roots, CB 47 89	Rio de Janeiro, Brazil	CNPAB collection
7	PRJ 14	Stem, SP 70 1143	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 17	Stem, IAC 52 150	Rio de Janeiro, Brazil	CNPAB collection
3	PRJ 20	Stem, Na 56 79	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 24	Roots, RB 73 9735	Rio de Janeiro, Brazil	CNPAB collection
7	PRJ 36	Stem, SP 70 1143	Rio de Janeiro, Brazil	CNPAB collection
7	PRJ 40	Leaves (trash), CB 36 14	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 54	Stem, Krakatau	Rio de Janeiro, Brazil	CNPAB collection
1	PRJ 56	Stem, SP 70 1143	Rio de Janeiro, Brazil	CNPAB collection
3	PRX 3	Xylem, CB 45 3	Rio de Janeiro, Brazil	CNPAB collection
3	PRX 6	Xylem, Na 56 79	Rio de Janeiro, Brazil	CNPAB collection
3	PSP 15	Roots, Na 56 79	São Paulo, Brazil	CNPAB collection
4	PSP 22	Leaf, Na 56 79	São Paulo, Brazil	CNPAB collection
3	PSP 32	Stem, Na 56 79	São Paulo, Brazil	CNPAB collection
6	PSP 17	Rhizoplane, Na 56 79	São Paulo, Brazil	CNPAB collection
3	PSP 19	Rhizoplane, Na 56 79	São Paulo, Brazil	CNPAB collection
1	URU	Roots, sugarcane	Uruguay	CNPAB collection
7	LMG 1733	Sugarcane	Australia	CNPAB collection
1	CFNE 530	Mealybugs-PT 49 143[b]	Veracruz, Mexico	This work
1	CFNE 531	Mealybugs-PT 49 143[b]	Veracruz, Mexico	This work
1	CFNE 532	Mealybugs-Z Mex 55 32[a]	Veracruz, Mexico	This work
1	CFNE 533	Mealybugs-L 78 56[b]	Veracruz, Mexico	This work
1	CFNE 534	Mealybugs-Mex 68 P23[a]	Sinaloa, Mexico	This work
1	CFNE 535	Mealybugs-RD 75 11[b]	Sinaloa, Mexico	This work
1	CFNE 537	Mealybugs-RB 73 9953[b]	Sinaloa, Mexico	This work
1	CFNE 539	Mealybugs-RB 72 1012[b]	Sinaloa, Mexico	This work
1	CFNE 541	Mealybugs-RB 72 1012[b]	Sinaloa, Mexico	This work
1	CFNE 542	Mealybugs-RB 72 1022[b]	Sinaloa, Mexico	This work
1	CFNE 544	Mealybugs-RB 73 9953[b]	Sinaloa, Mexico	This work
2	CFNE 550	Mealybugs-CB 45 3[d]	Rio de Janeiro, Brazil	This work
2	CFNE 554	Mealybugs-CB 45 3[d]	Rio de Janeiro, Brazil	This work
6	1772	Mealybugs	Ayr, Australia	M. W. Dawson[e]
1	PBD 4	Tuber, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
1	PBD 13	Peel, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
1	PBD 16	Roots, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
1	PBD 17	Tuber, sweet potato	Rio de Janeiro, Brazil	CNPAB collection
3	Pcol	Stem, *P. purpureum*	Rio de Janeiro, Brazil	CNPAB collection
7	PRC 1	Stem, *P. purpureum*	Rio de Janeiro, Brazil	CNPAB collection
7	PRC 4	Roots, *P. purpureum*	Rio de Janeiro, Brazil	CNPAB collection
3	7.10RM	Spores, VAM fungus[f]	Rio de Janeiro, Brazil	CNPAB collection

[a] Commercial sugarcane varieties.
[b] Sugarcane germoplasm.
[c] CNPAB, Centro Nacional de Pesquisa de Agrobiologia, Rio de Janeiro, Brazil.
[d] Collected from sugarcane grown in a concrete tank (5).
[e] M. W. Dawson, Sugar Research Institute, Mackay, Queensland, Australia.
[f] VAM, vesicular-arbuscular mycorrhizal.

recovered from different plants or mealybug colonies were considered to be different. Strain 1772 was kindly supplied by M. Dawson. Strains UAP 5560, PAl 5^T (= ATCC 49037^T [T = type strain]), and PPe 4 (= ATCC 49038) of *A. diazotrophicus*, corresponding to electrophoretic type (ET) 1, ET 3, and ET 4 as we described previously (8), were included as references in MLEE assays.

Culture media. *A. diazotrophicus* isolates and *Escherichia coli* HB 101 were grown in SYP medium (8) for all assays.

Preparation of cell extracts and MLEE. Each isolate was grown in 25 ml of SYP medium at 29°C and harvested by centrifugation, and pellets were suspended in 0.3 ml of 10 mM $MgSO_4 \cdot 7H_2O$ and treated as described previously (8).

Starch gel electrophoresis and the selective staining of 12 metabolic enzymes were done by methods described before (43). The enzymes assayed were the same ones used in a previous report (8), except for an unidentified dehydroge-

TABLE 2. Genetic diversity among isolates and ETs at 12 enzyme loci

Enzyme locus[a]	No. of alleles	Genetic diversity (H)[b] of:		No. of alleles	Genetic diversity (H) of:	
		55 isolates	7 ETs		49 isolates[c]	6 ETs[d]
IPO	1	0.000	0.000	1	0.000	0.000
LYD	2	0.197	0.285	1	0.000	0.000
LED	2	0.197	0.285	1	0.000	0.000
XDH	2	0.197	0.285	1	0.000	0.000
MDH	2	0.197	0.285	1	0.000	0.000
ADH	2	0.197	0.285	1	0.000	0.000
UDH	2	0.197	0.285	1	0.000	0.000
IDH	2	0.036	0.285	2	0.041	0.332
G6P	2	0.235	0.285	2	0.279	0.332
PGM	4	0.173	0.713	4	0.194	0.799
HEX	2	0.036	0.285	2	0.041	0.332
EST	3	0.103	0.523	3	0.119	0.600
Mean	2.16	0.147	0.316	1.66	0.056	0.199

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

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

[c] Excluding six isolates represented by ET 7.

[d] Excluding ET 7.

nase. This enzyme was visualized on gels stained for indophenol oxidase, which is revealed as white bands in the presence of light. In contrast, the unidentified dehydrogenase was observed as a typical purple band, like other dehydrogenases. We did not attempt to determine the substrate(s) for this enzyme. For all assays the electrophoretic buffer system used was Tris-citrate (pH 8.0). Distinctive combinations of alleles for the 12 enzyme loci (multilocus genotypes) were designated different ETs (43). The level of genetic diversity for each enzyme locus was calculated as described by Selander et al. (43).

Statistical analysis. The extent of linkage disequilibrium, or nonrandom association of alleles, in the studied population was evaluated to explore the degree of clonality. The ratio of the variance in mismatches observed (V_o) to the expected variance (V_e) in the population was iterated 10,000 times by a Monte Carlo procedure, as proposed by Souza et al. (47).

Total DNA isolation, DNA restriction, and filter blot hybridization. Total DNA was isolated as described previously (2). DNA was digested with *Eco*RI, and restriction fragments were electrophoresed, blotted, and hybridized as previously reported (8). DNA-DNA homology was based on relative levels of hybridization to ^{32}P-labelled DNA from *A. diazotrophicus* PAl 5^T. DNA amounts in gels and radioactivity levels were quantified as described before (26).

The restriction fragment length polymorphism patterns of the rRNA operons were determined from *Eco*RI DNA digests hybridized with a *Hin*dIII-*Hin*dIII 700-bp internal fragment from *E. coli rrnB* 16S rRNA genes cloned in pKK3535 (7).

RESULTS

MLEE and genetic diversity. A total of 55 isolates of *A. diazotrophicus* were examined, and 11 of the 12 enzyme loci analyzed were found to be polymorphic. The mean number of alleles was 2.16 (range, 1 to 4) (Table 2). A total of seven distinctive ETs were identified (Table 3). Most of the isolates (35 of 55; 64%) were identical, corresponding to ET 1. ET 4 and ET 5 were represented by only one isolate; ET 2 and ET 6 were each represented by two isolates only. Only strains corresponding to ET 1 were recovered from mealybug colonies associated with seven different sugarcane varieties and from 13 sugarcane varieties sampled in Mexico (including 5 Brazilian varieties); in contrast, seven ETs were identified from 7 sugarcane varieties cultivated in Brazil and from associated mealybugs (Table 1). Six of the ETs differed from one another at only one or two loci. However, strains grouped in ET 7 were very different from all other isolates, showing six unique alleles (Table 3). These strains were recovered from both *P. purpureum* and sugarcane sampled in Brazil, and one strain (LMG 1733) was isolated from sugarcane in Australia.

The mean level of genetic diversity per locus (H) among the seven ETs was found to be 0.316. However, the genetic diversity among isolates was lower ($H = 0.147$) (Table 2), reflecting the fact that four of the ETs were represented by one or two isolates, while only three ETs (ET 1, ET 3, and ET 7) represented 49 isolates (Table 3). Excluding ET 7, because it is largely divergent from all of the other ETs (see below), the H level among the six ETs was 0.199, and among isolates it was as low as 0.056 (Table 2), since in this case only two ETs (ET 1 and ET 3) represented 88% of the isolates.

The genetic relationships among the seven ETs are summarized by a dendrogram in Fig. 1. Six ETs (ET 1 to ET 6) were closely related, forming a cluster at a genetic distance of 0.195. A second line (ET 7), which contained six strains, was largely divergent, and it was separated by a genetic distance of 0.53.

DNA homology and ribosomal hybridization restriction fragment length polymorphisms. Six *A. diazotrophicus* strains from the closely related ETs 1 to 6 constituted a homogeneous group with relative levels of DNA homology ranging from 73 to 90% (mean homology, 86%) with reference strain PAl 5^T. This mean homology value was very similar to the level of 84% DNA homology previously determined by Gillis et al. (18) among three representative *A. diazotrophicus* strains, including the type strain PAl 5^T. The six strains corresponding to the more distant ET 7 (Table 1) exhibited only 54% homology to the same reference strain.

Strains CFNE 501, PAl 5^T, PSP 22, PAl 3, PRC 1, and LMG 1733 were analyzed by restriction fragment length polymor-

TABLE 3. Allele profiles at 12 enzyme loci in seven ETs of *A. diazotrophicus*

ET	Reference strain	No. of isolates	Allele at indicated enzyme locus[a]											
			IPO	LYD	LED	XDH	MDH	ADH	UDH	IDH	G6P	PGM	HEX	EST
1	CFNE 501	35	1	2	3	2	2	2	2	1	4	5	1	3
2	CFNE 550	2	1	2	3	2	2	2	2	1	4	6	1	3
3	PAl 5^T	8	1	2	3	2	2	2	2	1	5	5	1	3
4	PSP 22	1	1	2	3	2	2	2	2	2	4	5	2	3
5	PAl 3	1	1	2	3	2	2	2	2	1	4	7	1	1
6	1772	2	1	2	3	2	2	2	2	1	4	4	1	4
7	PRC 1	6	1	1	2	1	1	1	1	1	4	5	1	3

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

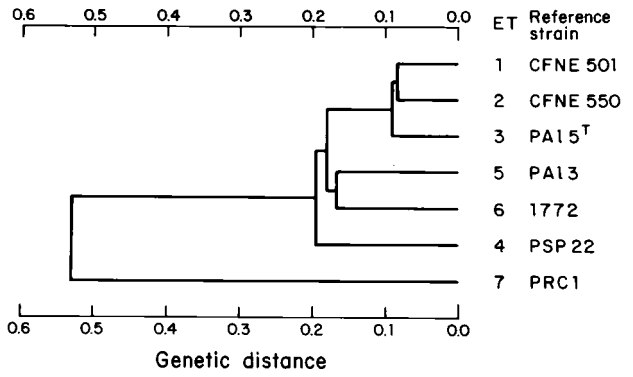

FIG. 1. Genetic relationships of ETs identified among *A. diazotrophicus* isolates recovered from different hosts.

phisms of the rRNA operons. The hybridizing patterns were identical. Four common hybridizing bands (9.3, 3.6, 2.3, and 1.6 kb) were observed in all of the isolates examined (Fig. 2). Similarly, all strains showed a common pattern (8) of hybridization to *nifHDK* (data not shown).

Linkage disequilibrium. A total of 1,485 pairwise comparisons are possible among the 55 isolates. The observed variance in proportion of mismatches was 5.806, and the expected variance was 1.450. The ratio of the observed variance in numbers of mismatches to the expected variance (V_o/V_e) was 4.003, highly significant, indicating a strong linkage disequilibrium. The analyses done separately for the populations recovered from sugarcane cultivated in Brazil and Mexico revealed a strong linkage disequilibrium also (data not shown).

DISCUSSION

In this study, we report that there is a lower genetic diversity in *A. diazotrophicus* recovered from different host species collected in widely separated regions of the world in comparison to the majority of other bacterial species studied (6, 9, 10, 13, 14, 19, 29, 35, 44). The results confirm previous data (8) on genetic diversity among 21 Mexican and 3 Brazilian isolates exclusively recovered from sugarcane plants. In addition, a new genetically distant group was found.

Coefficients of genetic distance at levels higher than 0.5 have been used as a criterion to suggest species limits (27, 45). DNA-DNA hybridization levels below 60 to 70% are also indicative of separate species (39, 48). On the basis of these facts, our MLEE studies suggest that the strains represented by ET 7 could represent a distinct nitrogen-fixing bacterial species. This result was consistent with the level of DNA-DNA homology obtained. In other cases, the estimates of genetic relatedness of strains obtained by both DNA-DNA hybridization and MLEE are closely correlated (24, 32, 41, 45). Nevertheless, restriction fragment length polymorphism patterns of ribosomal genes showed that ET 7 is related to the main *A. diazotrophicus* cluster. Due to the conserved nature of the 16S rDNA sequences, the method may be limited in the differentiation of closely related species (22). Furthermore, "DNA hybridization is acknowledged as the superior method for the elucidation of relationships between closely related taxa, such as strains and species" (48). Further work will be required to define the taxonomic status of ET 7 strains.

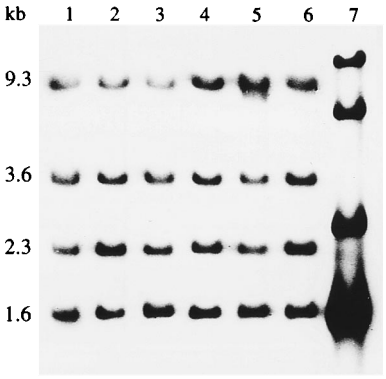

FIG. 2. Autoradiogram of a Southern blot of the total *Eco*RI DNA fingerprints hybridized with a 16S rDNA probe of *E. coli*. Lanes 1 through 6, strains CFNE 501 (ET 1), PAl 5^T (ET 3), PSP 22 (ET 4), PAl 3 (ET 5), PRC1, and LMG 1733 (ET 7), respectively; lane 7, *E. coli* HB 101, used as a control.

The restricted genetic variability observed in *A. diazotrophicus* suggests that this species has a recent evolutionary origin. Another possible explanation for the limited genetic diversity is related to the predominantly endophytic habitat of *A. diazotrophicus*, as suggested before (8), in association with niche specialization, because this species has been isolated exclusively from sucrose-rich host plants (15, 25) and from mealybugs associated with sugarcane plants (1). It has been postulated that each ecological niche acts as a selective force toward those properties of the organism that enable it to occupy that niche. Thus, the nonrandom variation suggests that the organisms are selected from those occupying closely related niches rather than very different niches (20). We do not discount that the limited genetic diversity observed in *A. diazotrophicus* could be related to the analysis of a limited subset of clones of the species, as advanced to explain the genetic diversity in other bacterial species (28, 45). This hypothesis is based on the very high selectivity of the medium used for bacterial isolation (15, 17, 36), which could influence the selection of subsets of all genotypes existing in nature. It has been observed that isolated soil bacteria make up only a very small proportion of the total bacterial community, but the largest proportion cannot be isolated or cultured on laboratory media (50).

Overrepresentation of a particular multilocus genotype is often the strongest and most significant evidence of clonality (49), particularly when the same genotype is recovered at many different localities and at different times (30, 45). The frequent recovery of isolates corresponding to the same ET from widely separated geographic regions, as well as from different hosts at different times, indicated that the genetic structure of *A. diazotrophicus* is basically clonal. This result was supported by the occurrence of a strong linkage disequilibrium in the natural population of these bacteria at both global and local levels.

The extensive distribution of closely related strains of *A. diazotrophicus* from widely separated areas of the world suggests that this bacteria was recently dispersed, as has been observed similarly in *Pseudomonas syringae* pv. tomato (14). Taking into account the endophytic characteristics of *A. diazotrophicus* and the association of the bacteria with mealybugs and vesicular-arbuscular mycorrhizal spores, we previously (8) explained the long-distance dispersal and spread among cane cultivars of this species.

It was previously suggested (1) that *A. diazotrophicus* may be autochthonous microbiota of mealybugs associated with sugarcane and other plants. However, we were able to isolate the bacteria from only 30 of 80 mealybug colonies of *S. sacchari*, including actively feeding adults, collected from stems of many different sugarcane varieties cultivated in both Brazil and Mexico. This fact suggests that *A. diazotrophicus* is sucked from sugarcane plants by the associated mealybugs, which is further

supported by our results showing that the *A. diazotrophicus* population recovered from *S. sacchari* is a subset of the *A. diazotrophicus* sugarcane population. The genetic diversity of *A. diazotrophicus* from other genera of mealybugs such as *Dysmicoccus brevipes* and a *Planococcus* sp. has not been analyzed, but perhaps it would be not surprising to find a limited genetic variability in these populations as well.

The comparison of the number of ETs identified in the collections of isolates recovered from sugarcane and mealybugs sampled in Brazil and Mexico showed that the population of *A. diazotrophicus* collected in Brazil, represented by seven ETs, is more heterogeneous genetically than the population collected in Mexico, represented only by ET 1. This apparent greater genetic heterogeneity may be related to the very different nitrogen fertilization levels that are applied to sugarcane field crops in Mexico in comparison to Brazil. A close relationship between nitrogen fertilization rates and isolation frequency of *A. diazotrophicus* was previously observed (17). At the highest fertilization rates (300 kg of N per ha), isolation frequencies of 0 to 2% were obtained, while at levels of 120 kg of N per ha frequencies increased up to 70%. Moreover, although Li and Macrae (25) did not mention any relation between isolation frequency of *A. diazotrophicus* and nitrogen fertilization, we noted that, in their results reported in Table 1, the number of isolates of this bacterium was nearly five times higher in the same sugarcane variety (CP 44101) with no N fertilizer than in N-fertilized plants collected in the same region and on the same date. Taking these observations into account, nitrogen seems to be a selective factor for certain lineages or clones of *A. diazotrophicus*. A role for a selective factor(s) may be supported in view of the endophytic nature of *A. diazotrophicus* organisms (25, 36), which supposedly are dispersed long distance inside sugarcane germoplasm commonly exchanged between countries (e.g., germoplasm of Brazilian varieties cultivated in Mexico [Table 1]). Therefore, in the absence of such a selective factor, different clones recovered in a country could be recovered from sugarcane germoplasm propagated in widely separated geographical areas. The nitrate levels available to the plant may increase or diminish the sucrose content depending on the sugarcane cultivar (34). This may explain to some extent the nitrogen fertilization effects on *A. diazotrophicus* populations, considering that sucrose is the best carbon source required in high concentration for optimal bacterial growth (11, 18). However, we could not exclude that other ecological factors, besides nitrogen fertilization rates, may contribute to the differences in genetic diversity of the *A. diazotrophicus* populations encountered in Brazil and Mexico.

Since a low number of isolates recovered from Cameroon grass and sweet potato were analyzed, it was not possible to determine if certain ETs of *A. diazotrophicus* are predominantly associated with a particular host species, as observed, for instance, with the pathogen of mammals *Bordetella bronchiseptica* (28) or with the legume-nodulating *Bradyrhizobium* sp. (6). However, the results clearly demonstrated that ET 1 was extensively distributed among all host species analyzed. From the viewpoint of biotechnological application, it will be important to determine if strains represented by the highly predominant ET 1 could be more efficient in promoting growth of the host plants by either involving indoleacetic acid (17) or supplying nitrogen (5, 51), or both, in comparison to other lineages, or if ET 1 is simply a highly "successful" lineage adapted to different host species.

Considering the apparent wide capacity of ET 1 to colonize sucrose-rich host plants, it will be interesting to determine this ability of ET 1 strains in other important sugar producer plants such as sugar beet (*Beta vulgaris*).

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