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Natural Endophytic Occurrence of *Acetobacter diazotrophicus* in Pineapple Plants

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ABSTRACT

The presence of endophytic *Acetobacter diazotrophicus* was tested for pineapple plants (*Ananas comosus* [L.] Merr.) grown in the field. Diazotrophic bacteria were isolated from the inner tissues of surface sterilized roots, stems, and leaves of pineapple plants. Phenotypic tests permitted the selection of presumptive nitrogen-fixing *A. diazotrophicus* isolates. Restriction fragment length polymorphisms (RFLPs) of small subunit (SSU) rDNA using total DNA digested with endonuclease *Sph*I and with endonuclease *Nco*I, hybridizations of RNA with an *A. diazotrophicus* large subunit (LSU) rRNA specific probe, as well as patterns in denaturing protein electrophoresis (SDS-PAGE) and multilocus enzyme tests allowed the identification of *A. diazotrophicus* isolates. High frequencies of isolation were obtained from propagative buds that had not been nitrogen-fertilized, and lower frequencies from 3-month-old plants that had been nitrogen-fertilized. No isolates were recovered from 5- to 7-month-old nitrogen-fertilized plants. All the *A. diazotrophicus* isolates recovered from pineapple plants belonged to the multilocus genotype which shows the most extensive distribution among all host species previously analyzed.

Introduction

Plant tissues are environments in which pathogenic bacteria may flourish. In addition, some specialized tissues are colonized by several symbiotic species related to the Rhizobia group. In the last few years, it has been recognized that healthy plant tissues are not necessarily free of bacteria, but can be inhabited by a diverse microbial community [17, 20, 25 28]. Some of those endophytic species have been shown to have positive effects on plant growth. Rice seedlings endophytically colonized by *Azoarcus* showed larger biomass increases than noninoculated controls [16]. This growth-promoting effect could be related either to the bacterial syn-

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thesis of hormone-like compounds or to biological nitrogen fixation [9].

The endophytic N_2-fixing species *A. diazotrophicus* was first isolated from sugar cane plants [5]. Several Brazilian sugar cane varieties have been shown to fix nitrogen [33], but the identity of the bacterium responsible is still unknown. It has been suggested that *A. diazotrophicus* contributes nitrogen to the plant [31], since it fixes N_2 in cultures within the osmolarity and acidity ranges prevailing in sugar cane tissues [5, 14]. *A. diazotrophicus* has been found to colonize sugar cane tissues that are devoid of dissolved carbon compounds, such as root and stem xylem vessels [18, 36]. The organism probably colonizes intercellular apoplastic stem spaces [8] that constitute the sucrose sink, and apparently also the phloem sieve tubes [10] that translocate this compound.

A. diazotrophicus colonizes the plant species *Saccharum* sp., *Pennisetum purpureum, Ipomoea batatas,* and *Coffea arabica,* which are localized in the taxonomic groups Poacea, Convolvulaceae, and Rubiaceae [5, 7, 19]. It has been suggested that the *A. diazotrophicus* association could be restricted to plants exhibiting vegetative propagation, since its known hosts employ this strategy. The maintenance of endophytic diazotrophic populations by plants could be advantageous for growth in soils with low fixed nitrogen. It has also been suggested that plant-associated endophytic bacteria have an essential role in the development of plants via the release of cytokinins [15]. *A. diazotrophicus* produces different plant hormones in culture media, including cytokinins and Indoleacetic acid [Jiménez-Salgado T, Caballero-Mellado J, Aparicio-Fabre R, unpublished data; 2; 11], which could promote the growth and development of its hosts.

The pineapple plant (Bromeliaceae) has several characteristics that are suitable for the endophytic growth of *A. diazotrophicus,* including high concentrations of carbon sources, acidic pH, and vegetative propagation. In addition, pineapple is commonly grown under climates within the temperature range that *A. diazotrophicus* requires. The aim of this work was to search for the presence of endophytic *A. diazotrophicus* in pineapple plants (*Ananas comosus* [L.] Merr.). Microbiological, biochemical, and molecular analyses of endophytic isolates from pineapple plants identified them as belonging to the N_2-fixing species *A. diazotrophicus.*

Methods

Media

N-free semisolid LGI containing 0.5% sugar cane juice [5] was used for the enrichment of *A. diazotrophicus.* LGI and potato agar [5] were used as morphology differential media for *A. diazotrophicus.* The oxidation of glucose and ethanol to CO_2 were determined in GYC and EYC culture media, respectively [6]. SYP broth [4], containing 3 g of yeast extract per liter was used for isolating RNA, total proteins, and obtaining extracts for multilocus enzyme electrophoresis (MLEE). Medium for testing lactic acid oxidation contained, per liter, 10 g yeast extract, 20 g lactic acid, 5 g $CaCO_3$, and 0.025 g bromcresol green, pH 6.0.

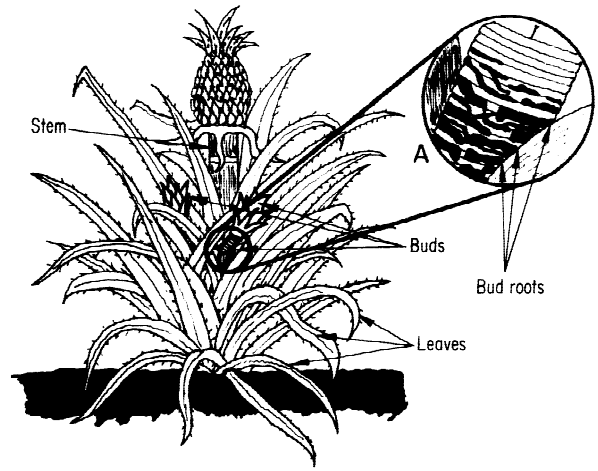

Fig. 1. Pineapple adult plant showing different organs and buds. Letter A represents a bud stem. The base leaves of the bud stem were not drawn to show the roots.

Isolation

A total of 19 pineapple plants (seven vegetative propagative buds, and five plants 3 months, two plants 5 months, and five plants 7 months old) were collected from Isla Municipe, in the Veracruz state, Mexico. Collection was done from two farms within a 20-ha area, to an approximate distance of 35–50 m between plants. Complete plants were washed with tap water and the bud roots exposed by removing the oldest leaves (Fig. 1). The roots, stems, and leaves were washed with sterile water and surface sterilized for 20 min with 5% calcium hypochlorite (Baker Inc.), then washed five times with sterile water. Thereafter, the samples were weighed and ground in a sterile mortar containing sterile sucrose solution (1%) in a ratio of 1:9 (w/v). Aliquots (500 µl) were inoculated in semisolid LGI [5] and incubated at 30°C for 4–6 days. Samples of the rhizosphere soil surrounding each pineapple plant were diluted (1:9) in 1% sucrose. Rhizosphere soil suspensions (aliquots of 100 µl) were inoculated in semisolid LGI medium and incubated as described above for plant samples. Fifteen replicates from each plant part (root, stem, leaves, and rhizosphere) were inoculated in semisolid LGI tubes. Yellowish bacterial growth from the tubes was streaked onto LGI plates [5] and incubated at 30°C for 6–7 days. The colony morphologies were compared with those of the *A. diazotrophicus* type and reference strains $PAl5^T$ (ATCC 49037) and UAP 5560 [11], respectively.

Phenotypic Characteristics

Acetylene reduction activity (ARA) was tested in tube cultures in semisolid N-free LGI medium. Isolated orange colonies on LGI plates derived from ARA positive tube cultures were selected to test for the production of dark brown pigment in potato agar, and for several biochemical tests [5, 12], such as catalase and oxidase activities, growth on different carbon sources, oxidation of ethanol and glucose to acetic acid and then to CO_2, and oxidation of lactate. Initial oxidation of glucose and ethanol were detected by the acidification and consequent dissolution of the precipitated $CaCO_3$ in GYC and EYC, respectively. The subsequent oxidation of the released organic acids produced an increase in the pH, again precipitating the $CaCO_3$ as described previously [5].

Multilocus Enzyme Electrophoresis

Starch gel electrophoresis and selective staining of 12 metabolic enzymes were performed by methods reported previously [30]. The enzymes assayed were the same ones used in a previous study [3] and were determined under the same conditions.

Total Protein Extraction and SDS-PAGE

Bacterial cultures were centrifuged at 8,000 × *g* for 10 min at 4°C and washed with 1% sucrose. The cells were resuspended in 2 ml of 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.05 M Tris-HCI, pH 7.4, and then lysed with three freezing cycles. The lysate was centrifuged for 30 min at 27,000 × *g* and the precipitate was discarded. The extracts were electrophoresed on SDS-PAGE gels (6% stacking gel and 12% running gel). The gels were fixed with methanol:acetic acid:water (3:1:6), stained in a filtered solution of ethanol:acetic acid:water (47.5:5.0:47.5) containing Coomassie Blue R (0.125%), and destained with methanol:acetic acid:water (3:1:6).

DNA Manipulation

Genomic DNA extraction and transfer to nylon filters was performed according to standard protocols [29]. A SSU rDNA fragment for probing was obtained by PCR amplification of genomic DNA with the primers fD1 and rD1 [35] that cover from position 8 to position 1541 of *E. coli.* PCR conditions were one cycle at 94°C (3 min), 35 cycles at 94°C (1 min), 57°C (1 min), and 72°C (2 min), plus one elogation cycle at 72°C (3 min). The probe was labeled with digoxigenin by random priming (Roche Molecular Biochemicals, Inc.). Blotted DNA, restricted with endonucleases *Sph*I and with *Nco*I, was hybridized at 65°C with an *A. diazotrophicus* SSU rDNA probe obtained from strain PAl5^T.

RNA Preparation and Hybridization

RNA was extracted from cells with the TriPure kit (Roche Molecular Biochemicals, Inc.) using the protocol suggested by the supplier. The RNA samples were blotted on nylon membranes, using 6 μg of RNA per slot. A DNA oligonucletide probe specific for the *A. diazotrophicus* LSU rRNA [13] was used for the hybridizations. The sequence of the probe was 5′-TGCGCCAAAAGCCGGAT-3′. The probe was labeled for 1 h at 37°C in T4 polynucletide kinase buffer (Roche Molecular Biochemicals, Inc.), using 20 pmol of oligonucleotide; 15 μCi [γ-^{32}P]ATP (specific activity >5,000 Ci/mmol, Amersham Pharmacia Biotech, Inc.); and 20 units of polynucletide kinase (Roche Molecular Biochemicals, Inc.). The hybridization conditions, kindly suggested by Dr. Kirchhof (Inst. Bodenkol., Munich), were as follows: 1 h prehybridization at 50°C in a solution containing (per liter) NaCl, 43.8 g; sodium citrate, 22 g; lauroylsarcosine, 10 g; Ficoll, 0.1 g; polyvinylpyrrolidone, 0.1 g; and bovine serum albumin, 0.1 g. Hybridizations were carried out overnight under the same conditions used for prehybridization. The nylon membranes were washed twice for 20 min at room temperature in a solution containing 0.3 M NaCl, 0.03 M sodium citrate, and 1% sodium dodecyl sulfate. A last washing step was held for 10 min at 50°C.

Results and Discussion

A. diazotrophicus is an endophytic bacterium that has been shown to colonize different plants. In this study, enrichment of pineapple endophytic bacteria in semisolid LGI allowed the recovery of 190 isolates that showed acidification, subsurface growth in N-free semisolid LGI, and acetylene reduction activity. Bacterial growth in the N-poor LGI agar medium produced acidification and *A. diazotrophicus*-typical colonies. The oxidation of lactate and the oxidation of ethanol to CO_2, catalase-positive and oxidase-negative activities, and the RFLPs of the SSU rDNA of total DNA restricted with endonucleases *Sph*I and *Nco*I (data not shown) indicated that all of the 50 *A. diazotrophicus*-presumptive isolates analyzed belonged to the family Acetobacteraceae [5, 6, 19, 32]. These isolates exhibited the oxidation of acetate, lactate and glucose to CO_2, growth and nitrogen fixation activity in N-free semisolid medium supplemented with 30% of glucose, and brown pigmentation of colonies grown on potato agar with 10% sugar. Bacterial growth was tested in the following carbon sources: galactose, arabinose, fructose, acetate, lactate, malate, ethanol, mannitol, glycerol, i-inositol, yeast extract, maltose, ribose, raffinose, rhamnose, and dulcitol. Results were consistent with their presumptive belonging to the species *Acetobacter diazotrophicus* [5, 6, 32]. Slot blotted RNA from all the *A. diazotrophicus*-presumptive pineapple isolates showed positive hybridization to an *A. diazotrophicus*-specific oligonucleotide probe ([13], Fig. 2) indicating they were a mem-

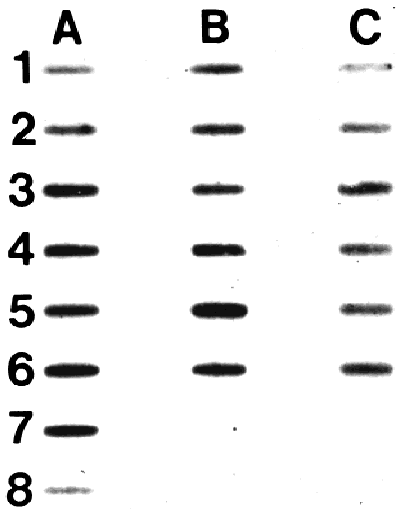

Fig. 2. Slot blot hybridization of RNAs of pineapple endophytic isolates with an *A. diazotrophicus* LSU rRNA specific probe [13]. *A. diazotrophicus* type strains PAl5^T (1A) and UAP-5560 (1B); pineapple isolates proceeding from distint regions of different plants UAP-Ac4 (2A), UAP-Ac3 (2B), UAP-Ac2 (2C), UAP-Ac7 (3A), UAP-Ac6 (3B), UAP-Ac5 (3C), UAP-Ac10 (4A), UAP-Ac9 (4B), UAP-Ac8 (4C), UAP-Ac25 (5A), UAP-Ac20 (5B), UAP-Ac15 (5C), UAP-Ac40 (6A), UAP-Ac35 (6B), UAP-Ac30 (6C), UAP-Ac41 (7A) and UAP-Ac43 (8A); other Acetobacteraceae species reference strains *A. aceti* ATCC 15973 (7B), *A. hansenii* ATCC 35959 (7C), *G. oxydans* ATCC 19357 (8B), and *A. liquefaciens* ATCC 14835 (8C). This autoradiogram represents a sample (9%) of the total number of isolates hybridized.

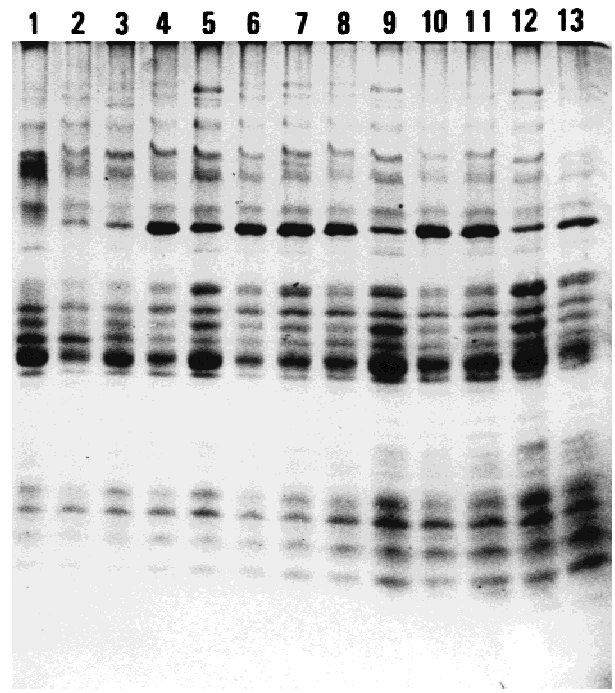

Fig. 3. Protein electropherograms (SDS-PAGE) of 11 endophytic isolates recovered from pineapple plants. *A. diazotrophicus* strains PAl5^T (line 1) and UAP 5560 (line 2); pineapple isolates UAP-Ac1 (line 3), UAP-Ac2 (line 4), UAP-Ac3 (line 5), UAP-Ac4 (line 6), UAP-Ac5 (line 7), UAP-Ac6 (line 8), UAP-Ac7 (line 9), UAP-Ac8 (line 10), UAP-Ac9 (line 11), UAP-Ac10 (line 12) and UAP-Ac15 (line 13).

ber of this nitrogen-fixing species. The phenotypic and hybridization identifications were confirmed by comparison of patterns of total proteins in electropherograms in SDS-PAGE gels. The SDS-PAGE patterns of 11 isolates recovered from different tissues of pineapple plants at diverse growth stages show very similar protein electropherograms among themselves and with the *A. diazotrophicus* type and reference strains (Fig. 3), indicating that all the strains analyzed constitute a homogeneous group of bacteria. Bacteria with very similar protein patterns are also considered to possess high genome similarity [34]. The slight differences observed in the protein electrophoresis patterns shown between the *A. diazotrophicus* strains PAl5^T and UAP 5560 could be related to their genotypic differences, detected previuously by multilocus enzyme electrophoresis assays [4]. Gillis et al. [12] also reported different protein patterns between some *A. diazotrophicus* strains, for instance, the strains PAl5^T and PR2. The strains UAP 5560 and PR2 were furtherly characterized and grouped in the same multilocus genotype [4].

Isolates with *A. diazotrophicus* characteristics were obtained in a range of frequencies from 0 to 80%, and the largest frequencies of isolation were obtained from non N-fertilized plants (Table 1). No *A. diazotrophicus* isolates could be recovered from rhizosphere soil. The isolation frequencies of *A. diazotrophicus* from pineapple propagules were similar to that reported in several sugar cane varieties cultivated with low rates of nitrogen fertilization [11]. The apparently diminished isolation frequency from adult pineapple plants that had been fertilized with nitrogen suggests that it has a negative effect in the establishment of *A. diazotrophicus* populations in this plant, as has been found in sugar cane [10, 11]. Nevertheless, an extended analysis of the nitrogen fertilization effect on the *A. diazotrophicus*–pineapple interactions is required because plant age could contribute to the results described above. In plant–bacteria interactions, it has been observed that the bacterial population fluctuates with time [25].

In this study, the multilocus enzymes tested in all of the *A. diazotrophicus* isolates recovered from pineapple plants showed the same electrophoretic mobility pattern (data not shown), which corresponds to the previously identified ge-

Table 1. Isolation frequencies of *Acetobacter diazotrophicus* recovered from pineapple plants[a]

Source	Roots	Stems	Leaves
Propagative buds	80	20	60
3-month-old plants[b]	20	nd	10
5 to 7-month-old plants[b]	nd	nd	nd

[a] Pineapple plants (cv. Smooth Cayenne) were sampled in the state of Veracruz, Mexico. The frequencies are expressed as the percentage of positive vials for isolation of *A. diazotrophicus.*
[b] Adult plants grown in fields under nitrogen fertilization for the time indicated.
nd indicates that no isolates were obtained.

notype ET1 [3, 4]; consequently this population shows a very limited genetic diversity. This correlates with the similarity in the patterns of protein electrophoresis of the pineapple isolates and of the reference strain *A. diazotrophicus* UAP 5560, which belong to the genotype ET1 [4]. In previous studies it was shown that one electrophoretic type (ET1) of *A. diazotrophicus* was distributed among all host species analyzed, including sugar cane, sweet potato, Cameroon grass, and coffee plants [3, 4, 19].

The genetic relationships of ETs identified among *A. diazotrophicus* isolates recovered from pineapple plants and from other host plants are summarized by a dendrogram in Fig. 4. The detection of one genotype (ET1) of *A. diazotrophicus* in taxonomically unrelated plants and the prevalence of this genotype in some of those plant species, as well as the very low genetic diversity in populations of *A. diazotrophicus* [3, 4], suggest several possibilities: (i) only some genetically related groups in this species or its ancestor have acquired the capability of colonizing plants by themselves or with the aid of vectors such as insects [1] or fungi [27]; or (ii) *A. diazotrophicus* had been associated with a particular plant taxonomic group, perhaps untested, and recently some selected genotypes have been able to extend its distribution to plants in other taxa.

It is proposed that the principal legume species have defined sites of origin and these coincide with the diversification centers for their symbiotic bacteria [24], either *Rhizobium* or *Bradyrhizobium.* The situation in the *A. diazotrophicus*–host plant associations is less clear. Originally, *A. diazotrophicus* was isolated from sugar cane [5, 11, 21], more recently from the coffee tree [19], and now from the pineapple plant. It is accepted that sugar cane is native to New Guinea [22] and coffee native to the Middle East [26], whereas the pineapple plant originates from the Amazonian region [23]. Since *A. diazotrophicus* shows more diversity

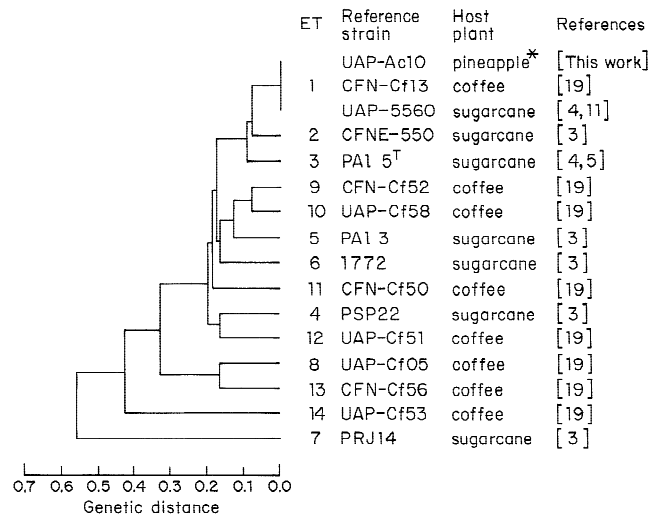

Fig. 4. Genetic relationships of ETs identified among *A. diazotrophicus* isolates recovered from pineapple, sugar cane and coffee plants. *All of the isolates recovered from pineapple plants were represented by the ET 1.

among isolates recovered from the coffee tree than from other hosts [3, 4], including the pineapple plants tested in this study, it could be suggested that the association of *A. diazotrophicus* with coffee is more ancient than its association with sugar cane or pineapple. On this basis, and considering the endophytic feature of *A. diazotrophicus,* it appears that the ET1 could have colonized the pineapple plant only recently, after the introduction of sugar cane and coffee trees to the Americas. The evolutionary trend of the populations of *A. diazotrophicus* could be established by analyzing a more extensive collection of strains isolated from different geographical regions, and different cultivars of diverse plant taxa and from the rhizosphere.

This study strongly supports the view that endophytic diazotrophic bacteria are more prevalent than previously believed.

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