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Natural endophytic association between *Rhizobium etli* and maize (*Zea mays* L.)

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Abstract

Maize (*Zea mays*) and bean (*Phaseolus vulgaris*) have been traditionally grown in association for thousands of years in Mesoamerica. From surface sterilized maize roots, we have isolated over 60 *Rhizobium* strains that correspond to *Rhizobium etli* bv. phaseoli (the main symbiont of bean) on the basis of 16S rRNA gene restriction patterns, metabolic enzyme electropherotypes, organization of *nif* genes, and the ability to nodulate beans. The colonization capacity of some of the isolates was tested with an unimproved maize cultivar and with 30 maize land races. Increases in plant dry weight upon *R. etli* inoculation were recorded with some of the land races, and these increases may be related to plant growth promotion effects. Additionally, from within maize grown in monoculture we have also recovered *R. etli* isolates recognizable by their 16S rRNA gene types, which lack *nif* genes and are incapable of nodulating bean. These strains are presumed to correspond to the earlier described non-symbiotic *R. etli* obtained from bean rhizosphere.

Keywords: *Rhizobium*; Endophytes; Maize; Land races; Nitrogen fixation

1. Introduction

Cereals such as maize have high N fertilization requirements for optimal yield. It would therefore be a noteworthy achievement if cereals could profit from biological nitrogen fixation and thereby decrease their requirements for N-chemical fertilization (Triplett, 1996). This goal could be attained by improving N_2 fixation by plant associated bacteria (Reinhold-Hurek and Hurek, 1998; James, 2000). In both sugar cane and rice, bacterial nitrogen fixation can contribute a substantial proportion of N to the plant (App et al., 1986; Boddey et al., 1991; Urquiaga et al., 1992; Watanabe et al., 1987). Multiple diazotrophic species and genera have been encountered inside rice plants (reviewed in Barraquio et al., 1997; Rao et al., 1998; James et al., 2000; Engelhard et al., 2000) and sugarcane (reviewed in Boddey et al., 1995; Baldani et al., 2000).

A review on the endophytic and rhizospheric bacteria of maize has been recently published (Chelius and Triplett, 2000b). Bacteria that have been identified as occurring inside surface steril-

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ized roots or stems include *Klebsiella pneumoniae* (Palus et al., 1996; Chelius and Triplett, 2000a), *Herbaspirillum seropedicae* (Olivares et al., 1996), *Burkholderia* spp. (Estrada-de los Santos et al., 2001), and *Arthrobacter globiformis* (Chelius and Triplett, 2000b). *A. globiformis* was identified as an endophyte in maize by a bacterial culture-independent approach. Endophytic colonization has been documented for *K. pneumoniae* (Chelius and Triplett, 2000a). The promotion of plant growth by *K. pneumoniae* does not appear to be related to nitrogen fixation (E. Triplett, personal communication). Similarly, the stimulation of maize growth by inoculation with rhizobia was found to be unrelated to N_2 fixation (Höflich et al., 1995). Acetylene reduction activity from maize plants and ^{15}N incorporation has been documented (Von Bülow and Döbereiner, 1975; Rennie, 1980; Alexander and Zuberer, 1989) but the bacteria responsible for this nitrogen fixing activity have not been identified as is also the case in sugarcane (Baldani et al., 2000).

Rhizobium spp. form nodules in legumes, providing fixed nitrogen to their hosts. Rhizobia have also been found to be capable of colonizing roots of non-legumes as efficiently as they colonize their legume hosts (Chabot et al., 1996 and references therein). In some cases, rhizobia have been shown to colonize non-legumes as endophytes. *Rhizobium*-non legume colonization may have been promoted by the ancient practice of legume-non legume crop rotation and in this context *Rhizobium leguminosarum* bv. trifolii was found to be a natural rice endophyte probably as a result of a 700-year-old tradition in Egypt of growing rice in rotation with berseem clover (Yanni et al., 1997). Moreover, *R. leguminosarum* bv. trifolii was found to promote the growth of rice plants (Yanni et al., 2000). More recent data show that even recently established legume-cereal rotation crops promote the endophytic rhizobium colonization of the wheat (Biederbeck et al., 2000) barley and canola (Lupwayi et al., 2000). *Azorhizobium caulinodans* colonizes the xylem of its host *S. rostrata* (O'Callaghan et al., 1999) as well as wheat (Sabry et al., 1997) and has been found to be a natural endophyte of modern rice cultivars (Engelhard et al., 2000). *A. caulinodans* survives in the soils and rhizosphere of wetland rice under *Sesbania rostrata*-rice rotation (Ladha et al., 1989). Penetration of rhizobia in non-legumes such as wheat, *Brassica*, and *Arabidopsis thaliana* does not require the *Rhizobium* nodulation genes which are involved in the infection and nodulation of legume roots (Gough et al., 1997; O'Callaghan et al., 1999), however, non-legume colonization by rhizobia is stimulated by flavonoids (Gough et al., 1997; Webster et al., 1998; O'Callaghan et al., 2000).

Maize and bean were domesticated in Mexico about 4000 years ago (Delgado et al., 1988; Gentry, 1969; Kaplan and Lynch, 1999). Traditionally, maize and bean have been grown in associations, called 'milpas' in Mexico, with the bean climbing over maize plants. In this system, the bean plant seems to enrich the soil N thus promoting the growth of maize. The advantages of bean-maize associations or intercrops have been recognized (Pineda et al., 1994; Souza et al., 1997). Nevertheless, the strategy that drove the Green Revolution was to grow both of the crops as monocultures and to select, in the presence of chemical fertilizer, high yielding varieties, that exhibit only a limited genetic diversity (Brush, 1986). However, 'milpas' subsisted in large areas of low income farming where they remain the best option for crop production. Due to the intimate bean–maize association in milpas, one might therefore expect to find *Rhizobium* strains as endophytes in maize. The aim of this study was to search for the presence of *Rhizobium* within maize roots. The *Rhizobium* strains obtained were characterized by a number of taxonomic criteria, which defined them as *Rhizobium etli*. Some of these isolates were subsequently examined for their endophytic re-colonization of maize.

In Mexico, *R. etli* bv. phaseoli is the main rhizobial species which effectively nodulates bean (Segovia et al., 1993). This species includes a large group of diverse bacteria, which contain a symbiotic plasmid with multiple copies of the *nif* genes (Martínez et al., 1985; Piñero et al., 1988).

2. Materials and methods

2.1. Strain isolation

Maize roots were washed with excess sterile distilled water after being surface sterilized with sodium hypochlorite (1.5%) for 15 min. Root-surface sterilization tests were performed by plating external root washings on plates with PY medium (0.3% yeast extract, 0.5% peptone, 0.07% $CaCl_2$ l^{-1}) or on YM medium (Vincent, 1970). To recover rhizobia from inside maize plants, roots were macerated and dilutions were plated on PY or YM media. Colonies appearing after 3 days were picked and streaked on PY Nal (nalidixic acid, 20 mg l^{-1}). Pearly, gummy bacteria that did not grow in LB (Luria–Bertani medium: 1% peptone, 0.5% yeast extract, 1% NaCl) were selected for further analysis. Otherwise, bean plants were used as traps to obtain rhizobia growing endophytically within maize and also to test if there were rhizobia at the maize surface after the sterilization procedure. Additionally, nodules from *Phaseolus* plants associated with maize were collected and rhizobia were recovered from these nodules as described by Martínez-Romero and Rosenblueth (1990). These rhizobia were designated bean rhizobia and all those coming originally from maize either directly on media or through bean selection were designated maize borne rhizobia.

Surface sterilized root macerates of maize were used as inocula for rootlets from pre-germinated bean seeds earlier surface sterilized as described (Martínez-Romero and Rosenblueth, 1990). Three bean plantlets were inoculated per maize root with 400 μl or aliquots of the macerate. Plants were maintained in growth chambers (28 °C). Ten days after inoculation, nodules on bean were counted, surface sterilized and crushed on plates. Ten nodules (large and small) per plant were streaked on PY media. Isolated colonies were further purified and maintained in YM stabs.

2.2. Multilocus enzyme electrophoresis (MLEE)

Extracts were prepared from each isolate grown on 30 ml PY liquid medium cultures, lysates and enzymatic activities were detected as reported by Selander et al. (1986). Gel electrophoresis was carried out in starch gels. The following metabolic enzymes were analyzed: hexokinase, phosphoglucomutase, isocitrate, malate, glutamate, and glucose-6-phosphate dehydrogenases, indophenol oxidase, and phosphoglucose isomerase. The different alleles (mobility variants) were designated according to mobility and electrophoretic types (ETs) were grouped from a pairwise matrix of genetic distances using the method described by Nei and Li (1979).

2.3. SSU rRNA gene typing

Almost complete 16S rRNA genes were synthesized with primers fD1 and rD1 (Weisburg et al., 1991). The PCR fragments were digested with *Msp*1, *Sau*3A1, *Hinf*1, and *Hha*1 and visualized in agarose gels as described (Laguerre et al., 1994). Patterns from maize borne rhizobia were compared with the type strains of bean-nodulating bacteria.

2.4. Plant growth promotion assays and colonization assays

Seeds of maize Puebla 162 (CIMMYT accession 19651, race Cacahu) or 'criollo' from Puebla were surface sterilized with ethanol 70% for 5 min and with sodium hypochloride 3% for 10 min prior to germination for 3 days on Whatmann filters with water. Individual seedlings were placed in tubes with 2 ml Fahraeus solution (Fahraeus, 1957) or in flasks with vermiculite moistened with Fahraeus solution and inoculated with 10^3–10^6 bacteria per plant in 0.1 ml of sterilized water. After 3 days in the dark at 28 °C, root and shoot weight and length were recorded from plants in tubes. Non-inoculated controls were treated only with the same amount of water and maintained under the same conditions. To evaluate endophytic colonization, 12-day-old plants in vermiculite flasks were surfaced sterilized as described before and macerates were diluted and plated in PY Nal plates. Endophytes were recovered and identified by their electrophoretic types. Colonization was also estimated from maize plants grown

in pots using the plant growth promotion assay as follows. In longer term experiments, seedlings sterilized from 30 maize races as described above were inoculated with 0.1 ml (per root) of bacterial suspensions in sterilized water containing around 10^6 bacteria per ml and then placed in 5 l pots, four plants per pot, containing sterilized vermiculite and soil (brown clay soil, pH 6.85) in a 3:1 ratio and maintained for 45 days in the greenhouse. The maize races used were Mich GP9 accession 10 race Connor, Mich GP13 accession 15 race Zamoam, Oaxa 48 accession 265 race Zapch19, Mexi 7 accession 1209 race Cacahu9, Mexi 48 accession 1398 race Chalqu9, Mexi 40 accession 1408 race Elotco, Mich 112 accession 1477 race Pepiti, Mexi 3 accession 2232 race Conico9, Mexi 526 accession 8521 race Elotco, Mich 115 accession 9116 race Connor, Mich 412 accession 9188 race Pepiti, Mexi 23 accession 10235 race Conico9, Mexi 35 accession 10432 race Chalqu9, Mich 5 accession 10496 race Zamoam, Mexi 319 accession 15777 race Connor, Mich 74 accession 16006 race Zamoam, Oaxa 684 accession 17901 race Bolita, Oaxa 686 accession 17903 race Bolita, Oaxa 695 accession 17912 race Bolita, Oaxa 704 accession 17921 race Bolita, Oaxa 742 accession 17959 race Bolita, Oaxa 803 accession 18020 race Bolita, Puebla 162 accession 19651 race Cacahu, Oaxa 331 race Blanco 2050, Oaxa 324 race Amaillo, Oaxa 153 race Blanco 1860, Oaxa 329 race Amaillo 2050, Oaxa 311 race Pinto, Oaxa 170 race Pinto 1900, Oaxa 323 race Negro.

2.5. nifH gene organization

DNA was isolated and digested with *Bam*H1 from representative maize borne isolates and reference *R. etli* strains. *nifH* fingerprint patterns were visualized by hybridization with a pEM15 300 bp internal *nifH* fragment from *R. etli* CFN42 as described (Wang et al., 1999).

3. Results

3.1. Bacterial isolation

Maize plants ($\approx$ 3-months-old) were collected in 1998 from a criollo maize in a traditional farmer's plot in Puebla, where maize was grown in association with bean and also in 1999 from Morelos, where maize was grown in monoculture. Isolates were also obtained from nodules of *Phaseolus vulgaris* (bean) grown in association with maize in 1998.

Endophytes are the microorganisms isolated from surface-sterilized plant tissues that do not cause any harm to the plant. It is difficult to be sure if an organism is a real endophyte or an outside survivor of an incomplete or inadequate surface desinfectation procedure. We verified that rhizobia were not survivors in the external rhizosphere either by using the external root washing as inocula for beans or by placing the sterilized root on YM media for either 20 min or 24 h, we did not obtain any nodules, nor any bacterial growth similar to *R. etli*, indicating no *R. etli* surface contaminants.

After surface sterilization of maize roots, using the macerates from maize roots and *Phaseolus vulgaris* bean as a plant trap, we isolated *Rhizobium* from the roots of all the five maize plants grown in association with bean but only from one of the five maize plants grown in monoculture. Around ten isolates per plant were retained. In the cases where no bean nodulation was recorded when using the maize root extract as the inocula (with the macerates from maize in monoculture), bacteria were recovered from the bean rhizosphere as earlier described (Segovia et al., 1991) and putative non-symbiotic *R. etli* were selected. In addition to *Rhizobium* we also encountered many other endophytic bacteria, which grow on PY or LB plates inoculated with maize root extract, however, the characterization of these bacteria was not pursued.

3.2. Bacterial identification

Rhizobium isolates were identified as *R. etli* strains by their ability to nodulate *P. vulgaris*, by their ribotypes, by the mobility of their metabolic enzymes in MLEE and by their organization of *nif* genes. All 63 maize rhizobia isolates formed red well-developed nodules in *P. vulgaris* (bean). All 63 isolates had identical ribotypes with pattern DBEC encountered also in *R. etli* strains CFN42, Bra5 (Piñero et al., 1988), CNPAF512 (Michiels et al., 1991), F8 (Wang et al., 1999) and Viking 1. This

pattern is different from those obtained with all the other reported *Rhizobium* species. Using MLEE, 12 maize borne strains were compared with 22 bean isolates from nodules obtained from bean associated with maize in the same agricultural field. MLEE analysis indicated that maize borne *R. etli* isolates were closely related to *R. etli* reference strains and to bean rhizobia (Fig. 1). From a single maize plant different ETs were recovered; for example, maize borne isolates CFNEM5-4, CFNEM5-9, and CFNEM5-3 from maize plant 5 (in association with maize) corresponded to ET3, ET4, and ET9, respectively, indicating the existence of different strains in a single plant. The *nif* gene organization was determined using 12 maize borne strains. They had reiterated *nifH* patterns characteristic of *R. etli* bv. phaseoli.

Ten putative non symbiotic isolates were assayed independently for bean nodule formation and from the analysis of their 16S rRNA patterns they were found to correspond to *R. etli* although their genomic DNA did not hybridize to *nif* gene probes (not shown). We presume therefore that these strains corresponded to the earlier described non-nodulating *R. etli* recovered from bean rhizosphere (Segovia et al., 1991).

3.3. Bacterial colonization

With 'criollo' maize at 12 days post-inoculation larger numbers (10^3–10^4 per root) of maize endophytic bacteria (CFNEM1-8, CFNEM5-1, CFNEM5-2, CFNEM5-3, CFNEM5-4, CFNEM5-5) were obtained compared with 10^1–10^2 bacteria per root for the *R. etli* reference collection strains, CFN42 and Bra5. Under these colonization conditions (flasks with vermiculite) there were around 1000-fold more bacteria of CFNEM1-8 on the root surface than inside.

A multi-strain inoculum consisting of the *R. etli* isolated from within roots of maize number 5 (from Puebla, isolates CFNEM5-1–CFNEM5-10) was used to inoculate plantlets derived from surface sterilized maize seeds; in this experiment, 30 maize native races were used. Plants which were maintained in a greenhouse were grown in pots with a mixture of sterile vermiculite and soil and were tested for the presence of *R. etli* within the roots 45 days after inoculation. Colony forming units of bacteria recovered as maize endophytes ranged from 10^2 to 10^6 per g root but in half of the races tested we found no *R. etli* colonization. No *R. etli* strains were found inside the non-inoculated maize plants used as controls. *R. etli* was found in the rhizosphere of most of the inoculated maize cultivars but was not quantified. We have identified some maize races (accession numbers 2232, 1398, 1408, 16006, 17901, 17912, 17921, 17959, 19651, Oaxa 170, Oaxa 329, Oaxa 331)

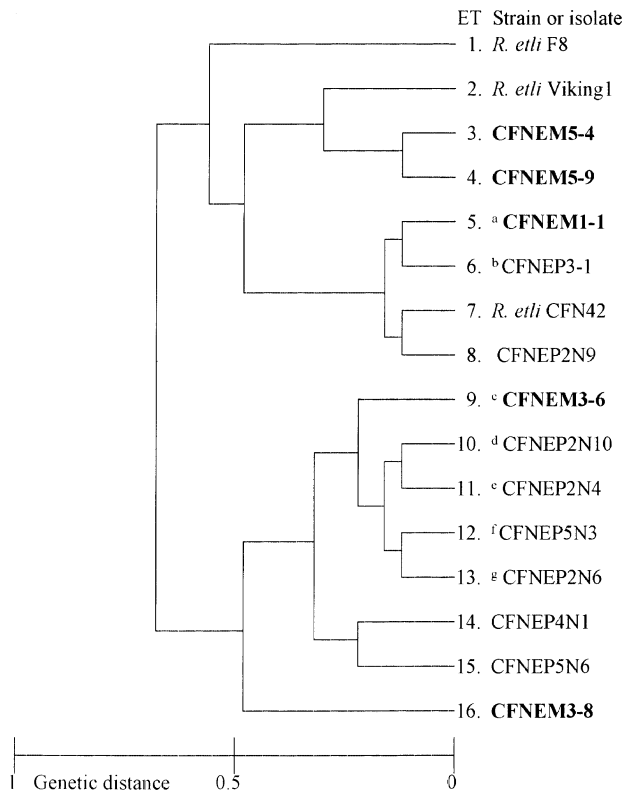

Fig. 1. Dendrogram of genetic distances deduced from metabolic enzyme electrophoretic patterns of *R. etli* reference strains, maize borne endophytic rhizobia and bean nodule isolates. All isolates from maize are bold. Other strains included per ET are: (a) **CFNEM1-10**, **CFNEM4A-2**, **CFNEM4B-1**, **CFNEM5-5**, **CFNEM4-1**, Bra5, CFNEP1N1, CFNEP2N5, CFNEP2N8, CFNEP4N2, CFNEP4N6, CFNEP4N9, CFNEP5N10; (b) CFNEP3-1N3, CFNEP3-1N4, CFNEP3-2N4; (c) **CFNEM5-3**, **CFNEM4A-1**; (d) CFNEP5N5; (e) CFNEP4N8; (f) CFNEP5N9; (g) CFNEP5N4.

Table 1
Maize Puebla 162 (3 days post inoculation) in tubes with Fahraues

Strain	Average per plant		
	Root weight (g)	Shoot weight (g)	Shoot length (cm)
CFNEM5-1	0.152 ± 0.013	0.380 ± 0.018	8.7 ± 0.27
CFNEM5-8	0.153 ± 0.012	0.328 ± 0.018	7.7 ± 0.37
CFNEM5-1 to CFNEM5-10	0.190 ± 0.016	0.390 ± 0.010	8.7 ± 0.20
CFN42	0.137 ± 0.009	0.360 ± 0.010	8.5 ± 0.22
CFNX249	0.140 ± 0.012	0.360 ± 0.030	8.7 ± 0.90
CNPAF512	0.110 ± 0.011	0.280 ± 0.035	6.4 ± 0.50
Non-inoculated control	0.110 ± 0.020	0.290 ± 0.031	6.5 ± 0.50

Three independent assays performed. One experiment is presented. Clear increases were obtained upon inoculation with *R. etli* borne isolates in all cases.

that show in all the plants tested increases of 10–100% over non inoculated controls with regard to plant dry weight upon inoculation with *R. etli* maize borne strains at 45 days after inoculation. Acetylene reduction assays were performed on whole maize roots but no activity was detected. We chose Puebla 162 for further analysis since it was the best responding maize race to *R. etli* inoculation. Net plant growth promotion effects on maize were observed upon *R. etli* inoculation in tubes (Table 1). The largest increases in root weight were recorded with the maize borne isolates. Positive effects on shoot weight and length were recorded with CFN42 and CFNX249 (Fix^- mutant, M. de L. Girard, unpublished) as well, but not with *R. etli* reference strain CNPAF512.

4. Discussion

In Mexico, soils with low nutrient availability are commonly used for maize production because production of high-value crops has displaced maize to marginal areas. N-fertilization rates in these areas are suboptimal since N fertilization constitutes a substantial cost to farmers. Following the rationale of Yanni et al. (1997), we surmised that rhizobia occurring inside maize could be more easily encountered in places where bean and maize have been associatively grown for thousands of years. In Tehuacan, Puebla, vestiges of 4000-years-old bean have been recorded (Kaplan and Lynch, 1999). We describe here that maize in milpas in Puebla normally harbor *R. etli*, the bacteria that form nitrogen fixing nodules in bean, and that these bacteria are naturally occurring maize endophytes. *R. etli* strains were encountered in all maize plants grown in association with bean but were found in only one out of five plants grown in monoculture. Maize borne *R. etli* showed the same *nifH* gene organization, the same restriction pattern of PCR-synthesized ribosomal genes and the same MLEE patterns as the isolates from *P. vulgaris* bean nodules. These findings imply that there is a common pool of *R. etli* chromosomal types in bean nodules and in maize. Plasmid borne traits may determine adaptation of *R. etli* to one niche or the other. Since *nod* genes are not required for non-legume colonization (Gough et al., 1997; O'Callaghan et al., 1999) and *R. etli* readily lose the *nod-nif* symbiotic plasmid (Segovia et al., 1991), it is not surprising that some *R. etli* strains recovered from within maize plants are non-symbiotic, these isolates do not possess *nif* genes and do not nodulate beans. The finding that symbiotic *R. etli* are more frequently obtained from maize grown in association with bean probably reflects the fact that symbiotic *R. etli* populations are promoted and enriched by the bean plants thereby constituting the source of inocula for maize plants.

Traditional agriculture has preserved land races. The maize races used in this study were

from diverse geographical regions in Mexico. It is worth noting that the best race responding to *R. etli* originates from the same geographical region where the strains were originally isolated, suggesting the existence of a local adaptation of maize plants and *R. etli*. The beneficial effects of *R. etli* on maize plant seem to be mediated by growth promotion as has been described earlier for *Rhizobium* (Chabot et al., 1996). *R. leguminosarum* bv. trifolii isolates from rice produce auxins and gibberellins (Yanni et al., 2000). The production of the bacterial mutualistic signal lumichrome that enhances root respiration, plant photosynthesis and growth has been reported (Phillips et al., 1999, 2000). We have detected that maize borne *R. etli* isolates CFNEM1-1, CFNEM5-1 and CFNEM5-8 produce lumichrome (E. Martínez, unpublished). However, it remains to be established if some beneficial effects may be derived from nitrogen fixation. An *R. etli* CFNX249 mutant with interposons in each of the *nifH* genes of the two *nif* operons has a Fix^- phenotype in bean (M. de L. Girard, unpublished). CFNX249 has been used as a nif^- reference strain of *R. etli* in other cases (Michiels et al., 1998; Brito et al., 1997; Cermola et al., 2000). We are testing *R. etli* mutant CFNX249 in long term plant assays.

It is notable that, as in other endophtyic associations (Yanni et al., 1997; Sabry et al., 1997; Fuentes-Ramírez et al., 1999), *R. etli* bacterial cell numbers (maximum 10^6 per gram recovered from the inoculation experiments using all of the land races) were not as high as those encountered in nodule-based nitrogen fixing symbiosis (reviewed in James, 2000).

From the acumulated data on the different systems studied, the following common features of the plant-endophytic diazotroph relationship seem to emerge: (i) the inside of roots is not an exclusive niche for a single bacterial species (in contrast to rhizobia nodules in legumes); (ii) the presence of endophytes and probably their role depend on the plant cultivar (Boddey et al., 1995) or domestication status of the species (Engelhard et al., 2000) as well as on the environmental conditions (Boddey et al., 1995; Chelius and Triplett, 2000a). (iii) Only a few plant cultivars from a given species seem to be favorable to support bacterial endophytic nitrogen fixation (Watanabe et al., 1987; Urquiaga et al., 1989; Boddey et al., 1995). (iv) Plant growth promotion may be due to mechanisms other than nitrogen fixation, such as hormone production (Sevilla and Kennedy, 2000; Yanni et al., 2000) or pathogen suppression.

Rhizobium as a model endophyte has the advantage in that it has been safely used as a plant inoculant in agriculture over a period of more than 100 years and it is readily amenable to genetic manipulation. We plan to pursue the analysis of the *R. etli* genes involved in the maize endophytic interaction with a view to enhancing the *R. etli*-maize mutualism. In addendum, we have found *R. etli* strains inside surface sterilized maize stems from field plants grown in association with beans.

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