Comparison of Benefit to Sugarcane Plant Growth and $^{15}\text{N}_2$ Incorporation Following Inoculation of Sterile Plants with *Acetobacter diazotrophicus* Wild-Type and *Nif*[−] Mutant Strains

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The ability of the nitrogen-fixing bacterial endophyte *Acetobacter diazotrophicus* strain PA15 to enhance the growth of sugarcane SP70-1143 was evaluated in the growth chamber, greenhouse, and field by comparing plants inoculated with wild-type and *Nif*[−] mutant MAD3A in two independent experiments. The wild-type and *Nif*[−] mutant strains colonized sugarcane plants equally and persisted in mature plants. In N-deficient conditions, sugarcane plants inoculated with *A. diazotrophicus* PA15 generally grew better and had a higher total N content 60 days after planting than did plants inoculated with mutant MAD3A or uninoculated plants. These results indicate that the transfer of fixed N from *A. diazotrophicus* to sugarcane might be a significant mechanism for plant growth promotion in this association. When N was not limiting, growth enhancement was observed in plants inoculated with either wild-type or *Nif*[−] mutants, suggesting the additional effect of a plant growth promoting factor provided by *A. diazotrophicus*. A $^{15}\text{N}_2$ incorporation experiment demonstrated that *A. diazotrophicus* wild-type strains actively fixed N_2 inside sugarcane plants, whereas the *Nif*[−] mutants did not.

Additional keywords: Acetobacteraceae, biological nitrogen fixation, diazotrophs, endophytes, grasses, meristem tissue culture.

Sugarcane production in many countries depends on the traditional practice of adding nitrogen fertilizer for optimum growth and yield. In regions of the world where N input is very low, the diazotrophic bacterium *Acetobacter diazotrophicus* (syn. *Gluconacetobacter diazotrophicus*) often has been isolated from surface-sterilized roots, stems, and leaves (Baldani et al. 1997; Bellone et al. 1997; Cavalcante and Do-

berreiner 1988; Dong et al. 1994; Fuentes-Ramirez et al. 1993; Li and McRae, 1991). It is considered to be an obligate endophyte because it has not been isolated from soil or rhizosphere regions in significant numbers, although it is cultured and manipulated easily in the laboratory. Results from ^{15}N isotope dilution and N balance experiments with several sugarcane cultivars indicate their ability to obtain significant amounts of atmospheric N through plant-associated biological nitrogen fixation (BNF) (Urquiaga et al. 1992). It was estimated that up to 80% of the total N of three sugarcane varieties, CB45-3, SP70-1143, and Krakatau (equivalent to 170 kg of N per hectare per year), can be attributed to BNF (Boddey 1995). The worldwide occurrence of endophytic *A. diazotrophicus* in sugarcane plants suggests a beneficial association between a diazotroph and a grass species. *A. diazotrophicus* also has become a strong candidate for the observed high level of BNF in sugarcane. In many previous studies of grass-associated diazotrophic bacteria, the observed plant growth promotion has never been unequivocally established to occur via transfer of fixed N from the diazotroph. In the case of the best-studied associative diazotroph, *Azospirillum brasilense*, evidence is strong that the production of the plant hormone indole acetic acid (IAA) is responsible for its ability to promote plant growth (Barbieri et al. 1991; Okon and Labandera-Gonzales 1994). This paper reports experiments aimed at determining whether *A. diazotrophicus* contributes significant fixed N to sugarcane. The wild-type strain PA15 and the *nifD* mutant were used to inoculate sterile sugarcane plantlets prepared from meristem tissue culture. Earlier results indicated a significant increase in the height of plants inoculated with the wild-type strain compared with the *nifD* mutant or uninoculated plants measured 30 days after planting in sterile sand supplied with N-deficient nutrient medium (Sevilla et al. 1998). In N-sufficient growth conditions, plants inoculated with either the wild-type or *nifD* mutant were approximately 20% taller than uninoculated plants. These results suggest that *A. diazotrophicus* can benefit sugarcane growth in two ways: by the transfer of bacterially fixed N as well as by the production of other plant growth promoting factor(s). These experiments now have been extended to include longer growth times and measurements of plant weight and total N incorporation. In addition, $^{15}\text{N}_2$ incorporation experiments were carried out

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and demonstrate that biological nitrogen fixation occurs inside inoculated plants but not in uninoculated plants or those inoculated with the *nifD* mutant strain. Results of experiments to examine longer term effects of *A. diazotrophicus* inoculated into sugarcane and the persistence of inoculated strains under field conditions also are included.

RESULTS

Effects of inoculation.

The effects of *A. diazotrophicus* on plant growth were assessed by measuring shoot and root mass and total N content of greenhouse-grown plants 60 days postinoculation (dpi). When N was limiting, sugarcane plants inoculated with wild-type strain PA15 had significantly greater shoot mass and N content than plants inoculated with mutant MAD3A or uninoculated plants (Fig. 1). In the first experiment, root mass and N content were significantly greater in plants inoculated with either wild-type PA15 or with MAD3A than in uninoculated plants. In the presence of excess combined N, plants inoculated with either the wild-type strain or the *Nif*⁻ mutant MAD3A had similar root and shoot mass and their N content was significantly greater than the values of uninoculated plants. Interestingly, roots and shoots of plants in experiment 1 responded much more to added combined N with respect to mass and N content than they did in experiment 2. For

uninoculated plants, the differences were 6.6- and 13-fold higher dry mass in roots and shoots, respectively, and 15- and 28-fold higher N content in roots and shoots, respectively, in experiment 1. In experiment 2, the responses to combined N were 3.1- and 4-fold higher in roots and shoots, respectively, in dry mass and 3.7-fold and 6.2 higher in roots and shoots, respectively, in N content. In addition, while the dry weight of roots and shoots was always higher in experiment 2 than in experiment 1, the total N content in plants supplied with combined N in the liquid medium supplied was higher in experiment 1 than in experiment 2. The reasons for this might reflect differences in growth rate and N partitioning in plants because of the different growth conditions in the two experiments. During experiment 1, the mean air temperature was 22°C and the average relative humidity (RH) was 27.3%. During experiment 2 the mean temperature was 30.5°C and the average RH was 48%.

In addition to total mass and N content, plant height, number of tillers, and number of leaves also were measured at 60 dpi. When N was limiting, plants inoculated with the wild-type strain were significantly taller and had more tillers and leaves than uninoculated plants, whereas plants inoculated with MAD3A did not differ significantly from uninoculated plants (data not shown). Consistent significant differences between inoculated and uninoculated plants were not observed in these parameters when fixed N was supplied to the plants.

Fig. 1. Mean plant biomass and total N content of sugarcane plants inoculated with *Acetobacter diazotrophicus* wild-type strain PA15 and *Nif*⁻ mutant MAD3A 60 days after planting. Number of plants analyzed for experiment 1, with -N and +N, respectively: uninoculated, 6 and 6; PA15, 10 and 8; MAD3A, 8 and 6. Number of plants for experiment 2, with -N and +N, respectively: uninoculated, 24 and 14; PA15, 24 and 13; MAD3A, 24 and 10.

Continued benefit of inoculation under field conditions.

To determine whether the observed growth enhancement could be sustained under field conditions, a number of greenhouse plants from experiment 1 were transferred to a field plot at random locations at 60 dpi, and these plants were grown for an additional 4 months without deliberate fertilization. Irrigation water supplied to this field at the University of Arizona Campus Agricultural Research Center (Tucson) contained amounts of fixed N from runoff sources that varied from day to day. Therefore, the fixed N supply was not controlled during the approximately 4 months that the sugarcane was growing in the field. Analysis of the fresh cane weight of sugarcane after that period indicates that plants inoculated with PA15 had greater mass than plants inoculated with MAD3A or uninoculated plants, whether or not N was supplied during the period of greenhouse growth before transplanting to the field (Table 1). These results indicate that the earlier benefits observed after growth chamber (30 days) (Sevilla et al. 1998) and greenhouse (additional 30 days) growth periods were sustained up to 4 months after transfer to field conditions.

Colonization of sugarcane plants.

A. diazotrophicus wild-type PA15 and the *nifD* mutant MAD3A colonized sugarcane plants equally, as evidenced by the number of bacteria recovered from inoculated plants 10 days after planting (dap) and 30 and 240 dap (Table 2). The same pattern of colonization also was found for these two strains. They were located mostly in the intercellular spaces of the roots and stems, although in some cases they also were present in the xylem vessels. *A. diazotrophicus* cells were commonly found in microcolonies inside the stems, with obvious clumping and a material that seemed to hold the cells together (Fig. 2). Bacterial cells often were found concentrated in the root cracks created by the emerging lateral roots, as observed by others (James et al. 1994). In the experiments reported here, entry could have occurred via these cracks or, more likely, through the openings at the tear-wound sites created by separating the plants before inoculation. Mutant and wild-type *A. diazotrophicus* also were reisolated from the sugarcane plants growing in the field at 240 dap. Bacteria in greenhouse plants were not counted because of the difficulty encountered in macerating plants at this stage of growth. Nevertheless, the characteristics of the isolated bacteria were always identical to *A. diazotrophicus* wild-type and mutant strains on the basis of the different tests mentioned.

The bacteria isolated from plants inoculated with MAD3A retained kanamycin resistance and were unable to grow in media without nitrogen (data not shown). Acetylene reduction

assays confirmed the inability of these mutants to fix dinitrogen. No difference was observed in the growth and morphology of reisolated bacteria from inoculated plants compared with the original bacterial inoculum. The identity of the isolated bacteria also was confirmed by the presence of a 400-bp amplification product with the use of 23S rRNA primers in all cases, wild type or mutant. Polymerase chain reaction (PCR) DNA amplification with uninoculated plants failed to yield any product. Immunogold labeling with antibody against nitrogenase showed that only the wild-type *A. diazotrophicus* expressed the enzyme inside sugarcane (data not shown). *A. diazotrophicus* was not isolated from the sand in growth chamber and greenhouse pots or field soil. Insects collected from the field also failed to yield *A. diazotrophicus* after plating on selective media (data not shown). No *A. diazotrophicus* were isolated from the uninoculated control plants at any growth stage.

¹⁵N-dinitrogen incorporation.

Sugarcane plants inoculated with wild-type PA15 incorporated ¹⁵N₂ into 0.4 and 0.2% of total N in shoots and roots, respectively, from nitrogen fixation over the 24-h period (Fig. 3), whereas the values for plants inoculated with the *nifD::uidA* mutant MAD3a were less than 0.05%, which is not significantly greater than what was found in the uninoculated plants. As might be expected, nitrogen fixation by wild-type PA15 was suppressed in the presence of combined N. Sugarcane inoculated with another wild-type strain of *A. diazotrophicus*, PPe4, also incorporated ¹⁵N₂ in an amount similar to that of PA15 under N-limiting conditions (0.5 and 0.1% in shoots and roots, respectively, of total N from nitrogen fixation), whereas a *nifD::uidA* derivative of PPe4, designated M4, gave less than 0.05%, again no greater than in the uninoculated control plants. The low ¹⁵N₂ values shown by uninoculated or Nif⁻ mutant inoculated strains are probably the result of small amounts of (¹⁵NH₄)₂ SO₄ not converted to ¹⁵N₂ by oxidation with hypobromite (see below).

DISCUSSION

Reports of enhanced growth of grasses in the presence of associative nitrogen-fixing bacteria are numerous. In no case was it shown that growth stimulation was caused by direct transfer of fixed nitrogen from the diazotroph to its plant partner. In this study, mutants specifically lacking the ability to fix nitrogen were used to evaluate the beneficial effects of *A. diazotrophicus* to sugarcane growth. Bacteria-free plants were used to demonstrate that *A. diazotrophicus* by itself can stimulate plant growth, confirming results of earlier studies with young sugarcane plants (Sevilla et al. 1998). More importantly, results of the present study indicate that the beneficial effects of *A. diazotrophicus* also can be demonstrated in more mature plants under controlled conditions and in the field.

Inoculation of sugarcane SP70-1143 with *A. diazotrophicus* consistently resulted in better growth of plants across experiments, although differences were not always highly significant in all the parameters measured. Under N-limiting condition, plants inoculated with PA15 grew significantly better than uninoculated plants, demonstrating the beneficial effects of inoculation with wild-type *A. diazotrophicus*. These results,

Table 1. Results of field experiment, 240 days after planting^z

Treatment	Fresh cane weight (kg)	Dry weight of green leaves (kg)
0 – N	5.499c	1.168b
PA15 – N	8.007ab	1.798ab
MAD3A – N	5.754c	1.270b
0 + N	6.379bc	1.543ab
PA15 + N	10.308a	2.359a
MAD3A + N	7.917ab	1.860ab

^z Means of total weight of canes or leaves per plant are given. Values shown in column followed by the same letter are not significantly different. Analyzed by Waller-Duncan method, *P* < 0.0002, α = 0.05.

combined with the observation that plants inoculated with MAd3A, were not significantly different from uninoculated plants, suggest that transfer of biologically fixed nitrogen under these well-defined N-limiting conditions is a likely reason for growth promotion. This is in contrast to other grass-associative bacteria like *Azospirillum brasilense* and *Azoarcus* sp., in which Nif⁻ mutants were not diminished in their ability to enhance the growth of tomato and rice seedlings, respectively (Bashan et al. 1989; Hurek et al. 1994). In this study, plants inoculated with Nif⁻ mutants were not always significantly different from wild-type inoculated plants and, in some cases, were significantly different from uninoculated plants, which suggests the involvement of another factor in addition to biological nitrogen fixation. The effect of this other factor is more pronounced when N is not limiting.

It also was apparent that more N was partitioned into the shoots than into the roots, especially under N-limiting conditions. In both experiments, the shoot N content of plants inoculated with wild-type PA15 was significantly higher than uninoculated control plants or plants inoculated with the Nif⁻ mutant MAd3A in N-limiting conditions. This effect was diminished when plants were supplied with N. In contrast, there were few significant differences found in the total N of the roots among plants in -N and +N conditions. Root lengths were not significantly different among treatments in the +N condition, but plants inoculated with wild-type strain PA15 had significantly longer roots than uninoculated plants. The Nif⁻ mutant MAd3A did not increase root length significantly compared with the control. Taken together, these data supply further evidence that nitrogen fixation is a benefit from *A. diazotrophicus* inoculation and further suggest that the production of other growth factors may be secondary to the effects of nitrogen fixation. IAA and gibberellins have been identified in cultures of *A. diazotrophicus* (Bastian et al. 1998; Fuentes-Ramirez et al. 1993), making either a candidate to influence plant growth. The recent report by Phillips et al. (1999) that lumichrome production by *Sinorhizobium meliloti* increases respiration and growth rate of alfalfa indicates another important class of microbial product that must be considered. Clearly the role of phytohormones and other sub-

stances produced by *A. diazotrophicus* in sugarcane growth promotion must be studied in future experiments involving mutants specifically lacking the ability to produce these compounds.

Variation in the measured parameters between the two experiments described may be a result of the differences in the two sets of meristem tissue-cultured sugarcane plants rooted at different times for each experiment. Although these plants probably represent a clonal population, morphologic, cytogenetic, and enzymatic variations occur frequently in sugarcane plants differentiated from tissue culture (Heinz and Mee 1971; Irvine 1984; Vasil et al. 1983). There were no gross morphological variations detected in the tissue-cultured plants used in this study, and similar-sized plantlets were used in all inoculation experiments. Nevertheless, it is possible that other variables may have induced the variation in plant responses observed in the two experiments. In addition, the plants were transferred in the greenhouse at different months, so fluctua-

Table 2. Number of bacteria reisolated from inoculated sugarcane

Treatment	Number of cells per gram of fresh weight [†]		
	10 dpi (MPN)	30 dap (MPN)	240 dap plate counts
0 - N [‡]			
PA15 - N	5.5×10^7	4.4×10^8	1.67×10^5
MAd3A - N	4.3×10^6	7.7×10^7	1.14×10^5
PPe4 - N	3.5×10^5	1.3×10^8	1.15×10^5
M4 - N	9.3×10^6	1.6×10^8	1.07×10^5
0 + N ^b			
PA15 + N	4.0×10^6	2.0×10^8	1.68×10^5
MAd3A + N	4.0×10^5	2.2×10^8	1.45×10^5
PPe4 + N	15.0×10^6	6.9×10^8	1.97×10^5
M4 + N	15.0×10^5	8.9×10^8	1.74×10^5

[†] Means of two experiments for 10 days postinoculation (dpi) and 30 days after planting (dap) and of one experiment for 240 dap. Bacteria were not counted at 60 dap because of the difficulty of macerating tissues. Isolated bacteria had the same characteristics as other isolated bacteria at different growth stages. MPN = most probable number estimates.

[‡] No bacteria were isolated from these treatments.

Fig. 2. *Acetobacter diazotrophicus* inside sugarcane 10 days after inoculation. Upper photo: transmission electron microscopy of *A. diazotrophicus* within the xylem vessel of sugarcane root. Lower photo: scanning electron microscopy of *A. diazotrophicus* cells inside sugarcane stem. Bacterial cells tend to clump and often are seen held together by a mucilaginous material.

tions in photoperiod and temperature could have affected growth, as reported for sugarcane (Yadava 1993).

Results of $^{15}\text{N}_2$ incorporation experiments also suggest a role for nitrogen fixation in the *A. diazotrophicus*–sugarcane association. To our knowledge, this is the first direct demonstration of biological nitrogen fixation by *A. diazotrophicus* inside sugarcane plants. The percent of ^{15}N excess values for plants inoculated with wild-type *A. diazotrophicus* were comparable with those obtained from two other tropical grasses with known associative nitrogen fixers, *Paspalum notatum* and *Digitaria decumbens* (De Polli et al. 1976; Dobereiner and Day 1976). The values are somewhat lower than those obtained for legume–rhizobia associations (Gil et al. 1997). Despite the short exposure time to $^{15}\text{N}_2$ in the experiment, the incorporation was nonetheless measurable and significant for plants inoculated with wild-type *A. diazotrophicus* strains. The question arises whether the $^{15}\text{N}_2$ was incorporated only into bacterial mass present inside the plants. Because no plant-specific compound was analyzed for $^{15}\text{N}_2$ content, we must rely on a theoretical consideration of the potential mass of

bacteria versus plant mass. The 15-dpi plants used here weighed approximately 0.5 g and contained approximately 10^8 bacteria, according to the “most probable number” (MPN) estimation measurements, which probably underestimate the true number of bacteria present. This number of bacteria (10^8) would have a protein content of approximately 100 μg (M. Sevilla, unpublished results), the N content being approximately 10 to 15 μg . Approximately 0.5 to 1% of the mass of plant material is N content and not measured in the 15-dpi plants, but indicated from the 60-dpi data (Fig. 2). In a plant mass of 0.5 g, 2 to 5 mg is N. If 0.3% of this is from BNF (average from Fig. 2), then the amount of $^{15}\text{N}_2$ would be approximately 15 μg , which is the same as that calculated to be the mass of $^{15}\text{N}_2$, which might be present in bacteria. While the data confirm that BNF was taking place in plants, it has not yet been proven that significant combined or fixed N was transferred from bacteria to plant material.

The results also showed higher ^{15}N incorporation in the shoots compared with the roots. This is in contrast to the results of Ruschel et al. (1971) in which fixed ^{15}N was detected in the separated roots but not the separated shoots of sugarcane seedlings. Because the bacteria associated with their sugarcane seedlings were not identified and were mostly detected in root epidermal cells, however, it is possible that the plants were not colonized by *A. diazotrophicus*. There was significantly higher ^{15}N incorporation in the plant roots inoculated with PA15 than in plants inoculated with PPe4, but no significant difference between them with respect to ^{15}N shoot incorporation. It is interesting to note that root sections of plants inoculated with PA15 always had higher bacterial concentrations than those from plants inoculated with PPe4, as judged by electron microscopy (M. Sevilla, unpublished). It can be argued that this result may be an artifact of sample preparation and could explain the lower ^{15}N incorporation observed in the roots of plants inoculated with PPe4. It has been reported that the isolation frequency of different Mexican strains of *A. diazotrophicus* vary within the stem of different sugarcane cultivars (Fuentes-Ramirez et al. 1993). It is possible that different *A. diazotrophicus* strains have preferred colonization sites inside sugarcane.

There were no differences observed in the ability of the wild-type and *Nif*[−] mutant to colonize sugarcane plants. Populations of similar size were isolated from inoculated sugarcane plants, even from plants grown in the field, suggesting that nitrogen fixation is not a prerequisite for endophytic growth within sugarcane. In contrast, *Nif*[−] mutants of *Azoarcus* spp. lost the ability to persist in the rhizosphere and could not colonize Kallar grass as well as wild type (Hurek et al. 1997). In the present study with the *nifD* mutant, MAD3A was found at the same concentration inside the plant as was wild-type strain PA15 and retained kanamycin resistance (Table 2). Acetylene reduction assay and $^{15}\text{N}_2$ incorporation showed that MAD3A was unable to fix nitrogen in culture and in planta.

The fresh cane weight of the harvested sugarcane plants revealed the continued growth benefit of early inoculation with wild-type *A. diazotrophicus* but not with the *Nif*[−] mutants when plants were pregrown in either −N and +N conditions. Specifically, inoculation with the wild-type PA15 resulted in significantly heavier canes compared with uninoculated plants regardless of the N condition. The dry weight of the green leaves of all field-grown sugarcane plants did not vary signifi-

Fig. 3. $^{15}\text{N}_2$ incorporation in the roots and shoots of sugarcane plants inoculated with *Acetobacter diazotrophicus* wild-type strains (PA15 and PPe4) and *Nif*[−] mutants (MAd3A and M4) grown with (+) or without (−) added mineral N. 0 = uninoculated.

cantly among treatments. Plants inoculated with PA15 had the highest dry weight of green leaves in both N treatments in the two experiments. Although a limited number of plants were examined in this field experiment and the plants were not grown to full maturity, the data indicated that the benefits of *A. diazotrophicus* inoculation through biological nitrogen fixation and other mechanisms can be utilized in field cultivation of sugarcane. This is an important consideration because, in many cases, the efficacy of a bacterial strain in the laboratory and greenhouse are often diminished when tested in the field. It will be interesting to see the effect of inoculation on the first and second ratoons in which response to the N application were shown to be more dramatic than plant cane response (Wiedenfeld 1997).

The reisolation of *A. diazotrophicus* wild-type and mutant strains from inoculated field-grown sugarcane demonstrated the persistence of this organism within sugarcane plants. *A. diazotrophicus* was not isolated from the soil in the field or from insects captured in the sugarcane field (data not shown) nor from uninoculated plants. This is not surprising because the only confirmed way of natural spread of *A. diazotrophicus* is through vegetative propagation of setts (Dong et al. 1994).

In summary, we have shown that inoculation with *A. diazotrophicus* enhanced the growth of sugarcane variety SP 70-1143 in growth-chamber, greenhouse, and field conditions. One mechanism of plant-growth enhancement probably is the transfer of biologically fixed nitrogen from the diazotroph to sugarcane as evidenced by the significant growth difference between plants inoculated with wild-type strains and Nif⁻ mutants under N-deficient conditions, and the similar results obtained with Nif⁻ mutants and uninoculated plants. Also, only the plants inoculated with wild-type strains were able to incorporate significant ¹⁵N₂. Because there were cases in which the difference in growth enhancement between mutant and wild-type inoculations were not significant, however, the involvement of other factors cannot be excluded. The effect of other factor(s) was more pronounced when N was not limiting. We do not discount the possibility that an interaction between nitrogen fixation and these other factors may be responsible for the improved plant growth.

MATERIALS AND METHODS

Plant material and bacterial strain culture conditions.

Micropropagated sterile sugarcane plants of cultivar SP70-1143 were obtained by meristem tissue culture as previously described (Sevilla et al. 1998). SP70-1143 is a commonly grown sugarcane cultivar in regions of Brazil with N-deficient soils. It can produce high yields even with low N fertilization. The *A. diazotrophicus* wild-type strain used was PA15, originally isolated from the roots of sugarcane growing in Alagoas, Brazil. MAd3A is a *nifD::aph* mutant of PA15 (Sevilla et al. 1997; Sevilla et al. 1998). PPe4 is another wild-type strain of *A. diazotrophicus* isolated from the stems of sugarcane growing in Pernambuco, Brazil. A Nif⁻ mutant of PPe4, M4, also was constructed in the same way as MAd3A. Cultures of wild-type and Nif⁻ mutant strains were grown in DYGS broth (Stephan et al. 1991) for 24 h at 30°C. Bacterial inoculum was prepared by centrifugating the cells from culture, washing three times with 25% LGI salt solution (no sucrose) (Cavalcante and Dobereiner 1988), and resuspending in the

salt solution to an optical density at 600 nm of 0.6 (approximately 1.5×10^8 cells per ml).

Inoculation of sterile sugarcane plants and assessment of colonization and plant growth.

Two independent inoculation experiments were performed in which the growth of the plants and/or bacterial colonization were monitored at different stages: 10 dpi in baby-food jars, 30 dap in a growth chamber, and 60 dap in a greenhouse. In experiment 1, plants were transferred to the field and harvested at 240 dap. The experiments were conducted throughout 1998. In experiment 1, the date of inoculation was 7 March, 1998. Plants were inoculated on 10 June, 1998, in experiment 2.

Inoculation was performed with the method of James et al. (1994) with some modifications. Two days before inoculation, rooted plants of uniform height (approximately 6 cm) and root mass were washed with N-free MS medium (Murashige and Skoog 1962) and transferred into fresh MS medium without N. On the day of inoculation, the plantlets were separated by tearing into four to five individual subplantlets of approximately equal root mass. These were placed in baby-food jars containing 40 ml of MS medium, with or without ammonium nitrate (4 mM). One-hundred microliters of wild-type or mutant bacteria (approximately 10^7 cells) was added to the medium for inoculation. Control plants received 100 µl of LGI salt solution. Plants were chosen at random for inoculation. Inoculated plants were kept on growth shelves at 28°C with a 12-h light and dark diurnal cycle. Ten days after inoculation, plants from each treatment were examined for colonization by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and MPN estimation (Postgate 1969).

Ten days after inoculation, sugarcane plantlets were transferred to autoclaved silica sand (grain no. 20) in conical containers sterilized with bleach. The plants were incubated in a growth chamber at 28°C with 12-h light and dark cycles. During the first two days of transfer in the growth chamber, plants were covered with clear plastic bags to maintain high humidity. Plants were watered with 40 ml of MS medium once every 2 weeks with and without combined N and with 40 ml of sterile distilled water as needed (approximately every other day). Thirty days after inoculation, MPN bacterial counts were measured. Bacteria isolated from inoculated plants were tested by growing in LGI medium (10% sucrose, pH 4.5) with or without kanamycin and by testing their ability to grow in N-free media and to reduce acetylene to ethylene. In addition, *A. diazotrophicus* specific primers were used in PCR reactions to amplify the 23S rRNA of isolated bacteria (Kirchof et al. 1988).

The plants were transferred 30 dap to 4.55 liters of bleached and washed pots containing sterilized silica sand. Pots were randomly arranged on benches in the greenhouse and watered by automatic drip irrigation twice each day. Once each week, 250 ml of half strength MS nutrient solution, with or without N (4 mM ammonium nitrate), was added to each plant. After an additional 30 days of growth, plants were harvested and separated into roots and shoots (numbers of plants for each experiment and treatment varied) (Figs. 1 to 3, legends). The separated parts were dried to a constant weight in a 70°C drying oven. Total N content of the plants were determined with an NCS analyzer (Carbo Erba NA1500), and the process

performed by the Soil and Plant Analysis Lab of the University of Arizona. Two plants from each treatment were used for SEM, MPN counts, and other identification tests.

Seven plants from each treatment in the first greenhouse experiment were transplanted to the field at the Campus Agricultural Center. Approval for this field trial was provided by the University of Arizona Biosafety Committee and the Arizona Department of Agriculture after considering the low risk that the *Nif⁻* mutant would pose to the environment. The field plot has sandy clay loam with available nitrogen content (NO_3) of approximately 2.3×10^7 mg of soil per kg. The experimental field was planted with a completely randomized design. Plants were spaced 76.2 cm apart within rows and the distance between rows was 121.9 cm. The field was flood irrigated once a week with no further addition of fertilizers, although the water for irrigation was run off from other experimental station areas and was neither controlled nor examined for fixed N content. Therefore, in the field experiments there was no control for a fixed N supply. The plots were routinely checked for insects and disease symptoms as well as damage by animals. Soil samples and insects were periodically taken from the field plots and checked for the presence of *A. diazotrophicus*. In addition, the presence of kanamycin-resistant bacteria from the soil, insects, and plant debris were monitored to comply with the requirements of the Biosafety Committee. There were no kanamycin-resistant bacteria isolated from the samples taken at different time points. The field also was left fallowed after the trial. The plants were harvested after 6 months, and fresh cane weight and dry weight of the remaining top green leaves were measured.

Statistical analysis of plant-growth experiments.

Data were transformed with the log function before performing statistical analysis. Analyses were performed with the PROC GLM procedure of the SAS computer program (SAS Institute, Cary, NC, U.S.A.). Mean separations were determined by the Waller-Duncan *k* ratio *t* test (*k* ratio = 100). Data were analyzed for each date and experiment separately.

Analysis of bacteria reisolated from inoculated plants.

To determine the concentration of *A. diazotrophicus* mutant and wild-type strains inside sugarcane plants in each stage of the experiment, MPN and plate counts were used. Plants were washed twice in distilled water to remove soil and other debris adhering on the surface. Plants were sectioned to obtain portions representing roots, stems, and leaves with fresh weight of approximately 1 g. Tissue-cultured plants were surface sterilized with 1% chloramine T for five min followed by three washes with sterile distilled water. Other plants were surface sterilized by soaking in 70% ethanol for 2 s and 10% bleach for 10 min, also followed by three washes in sterile distilled water with 5 min per wash. Plant tissue samples were macerated with a glass tissue homogenizer or Waring blender (East Windsor, NJ, U.S.A.) in 9 ml of distilled water or 25% LGI salt solution until no tissue particles greater than 2 mm were present. The resulting homogenate was serially diluted in 25% LGI salt solution. One hundred microliters of the diluted homogenates were either spread onto LGI plates or inoculated into glass vials containing 6 ml of semisolid LGI medium with or without N and with or without kanamycin. For MPN counts, three vials for each dilution were inoculated with three

replications for each dilution and treatment. All inoculated media were incubated at 30°C for 5 to 7 days. Colonies growing on the plates were counted and positive vials showing typical *A. diazotrophicus* pellicle and growth were scored with the McGrady tables (Postgate 1969).

PCR with the species-specific primer pairs AD and 1,440 derived from the variable region of *A. diazotrophicus* 23S rDNA were performed as described in Kirchoff et al. (1998) with some modifications. DNA was directly amplified from colonies growing on DYGS or LGI plates. One-day-old colonies were transferred with a toothpick to a microfuge tube containing 40 μ l of sterile distilled water. The bacterial suspension was boiled for 10 min and 2 μ l of the suspension was mixed with the other PCR reactants in PCR tubes. The amplification products were analyzed by electrophoresis in 0.8% agarose gels. Sugarcane plants also were examined for the presence of *A. diazotrophicus*. Plants were cut into small sections and macerated in sterile distilled water with a glass homogenizer, filtered, and centrifuged as described in Kirchoff et al. (1998) before they were used as templates in PCR reactions with conditions similar to that of bacterial amplifications.

Electron microscopy.

Sugarcane plants were hand sectioned to obtain 2 to 3 mm² segments. For SEM, root, stem, and leaf sections were fixed separately in a solution containing 4% formaldehyde and 1% glutaraldehyde (vol/vol) in 0.1 M phosphate buffer, pH 7.2, for 24 h (Trump 1978). The sections were then rinsed in 0.15 M phosphate buffer twice before postfixation with 1% (wt/vol) osmium tetroxide for 1 h. Samples were rinsed in distilled water three times and stained in 2% uranyl acetate for 30 min prior to dehydration in a graded ethanol series. The plant segments were freeze fractured in liquid nitrogen and critically point dried in a Polaron critical point drier (Energy Beam Sciences, Agawam, MA, U.S.A.) prior to mounting and coating with gold in Anatech Technics Hummer 1 sputter coater. Sections were examined under SEM (WB6, Topcon, Paramus, N.J., U.S.A.) operating at 10 kV. Some plant materials were fixed and prepared as above but with less time with the use of a 34350 laboratory microwave processor (Ted Pella, Redding, CA, U.S.A.) as outlined in Gibberson et al. (1997).

Sugarcane plants for TEM were treated the same way as for SEM except that plant segments were fixed in 0.1 M phosphate buffer with 2% formaldehyde and 0.5% glutaraldehyde (vol/vol). After dehydration in the ethanol series, segments were embedded in LR White acrylic resin with 2 days infiltration, followed by curing at 75°C for 8 h. Sample preparation and immunogold labeling were performed with the microwave technique (Gibberson et al. 1997) as described for SEM. Sections were collected on pyroxylin-coated nickel grids and stained for 10 min in uranyl acetate before being viewed with a Philips 420 TEM.

¹⁵N incorporation.

Thirty day postinoculation sugarcane plants were transferred to sterile 1 $\times$ 8-in. test tubes containing 30 ml of sucrose-free MS media with or without N. All plants, including the uninoculated control plants, were approximately 8 cm tall. The roots of the plants were washed several times with N- and sucrose-free MS media before transfer into the test tubes. The tubes were closed with a rubber stopper with a hole fitted with

a Pasteur pipette, with one end penetrating the rubber stopper into the test tube and the other extending into the top of the tube. The end extending to the top of the tube was fitted with another rubber stopper. A small vial containing 200 µl of 0.001 M NaHCO₃ with cresol red as indicator was suspended from a string in the test tube. Initially after the plants had been transferred to the tubes, the tubes were closed and air was evacuated through a Pasteur pipette with a syringe fitted with a needle. An atmosphere containing 20% O₂, 65% He, and 15% N₂ enriched with ¹⁵N was added into the test tube. This is equivalent to 10 ml of O₂, 32 ml of He, and 8 ml of ¹⁵N₂. The ¹⁵N₂ gas was generated in a syringe by oxidizing ¹⁵(NH₄)₂SO₄ (62.9 atom% ¹⁵N) with hypobromite made by reacting bromine and 5 N NaOH. The generation of ¹⁵N gas was checked by running a sample of the generated gas in a gas chromatography mass spectrometer. CO₂ (0.2 ml) was then added to the test tube, and the vial was jiggled. When CO₂ is virtually exhausted in the system, the cresol red indicator turns red, whereas in the presence of sufficient CO₂, the indicator turns yellow. The entire setup was incubated for 24 h on a growth shelf, and the CO₂ was replaced as needed based on the indicator color. After 24 h, the plants were washed several times in sterile distilled water, separated into roots and shoots, dried in the oven, and ground. Samples were subjected to Kjeldahl digestion, the digests were made alkaline, and the ammonia released was distilled into dilute H₂SO₄. Each distillate was concentrated to approximately 1 ml and was converted to N₂ in an evacuated tube with lithium hypobromite. The N₂ was analyzed for ¹⁵N in an isotope ratio mass spectrometer (Burris 1972; Burris and Wilson 1957).

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