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Contributions of the Bacterial Endophyte *Acetobacter diazotrophicus* to Sugarcane Nutrition: A Preliminary Study

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Abstract

The nitrogen-fixing bacterial endophyte *Acetobacter diazotrophicus* has been proposed to benefit sugarcane growth. In order to quantitate this benefit and determine the relative contribution of bacterial nitrogen fixation and/or other factors, a genetic approach was initiated. The complete sequence of the structural genes encoding the nitrogenase enzyme, *nifHDK*, has been determined. The *nifHDK* gene products are highly similar to NifHDK of other plant-associating bacteria in the α-subgroup of Proteobacteria. Mutants with insertion in the *nifD* region were unable to fix nitrogen (Nif$^-$). These Nif$^-$ mutants could colonize plants and proliferate within tissues to the same extent as wild type bacteria. Initial experiments showed growth stimulation in young sugarcane plantlets inoculated with wild type but not with Nif$^-$ mutants under N-deficient conditions. Interestingly, when N is not limiting, both wild type- and mutant-inoculated plants showed increased growth compared to uninoculated control plants suggesting other possible beneficial effects of *A. diazotrophicus* to sugarcane in addition to nitrogen fixation.

Keywords: *Acetobacter diazotrophicus*, nitrogen fixation, nitrogenase, endophyte, *nifHDK*, sugarcane

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1. Introduction

Availability of fixed nitrogen is the most significant yield-limiting factor in many agricultural production systems. With the increasing cost of chemical fertilizers and concern with environmental pollution, the role of biological nitrogen fixation (BNF) in supplying plants with needed N has become more important than ever. The most well-known nitrogen fixers are species of *Rhizobium* and of the related genus, *Bradyrhizobium*. The association of rhizobia with legumes is well-documented and represents the most important and established symbiosis in agriculture (Boonjawat et al., 1991). The potential of extending this symbiosis to other plants especially in important monocot crops like rice have been explored for many years and continue to be the focus of many research programs (Khush and Bennett, 1992). The isolation of naturally occurring nitrogen-fixers in cereals and grasses has also been explored. In particular, Döbereiner and her co-workers in Brazil have isolated a number of diazotrophic bacteria from tropical grasses including species of *Azospirillum*, *Herbaspirillum*, and *Azotobacter* (Döbereiner and Day, 1975; Döbereiner et al., 1988). The most intriguing of these bacteria is *Acetobacter diazotrophicus*, a gram-negative, rod-shaped, acid-tolerant bacterium associated with sugarcane. Other interesting characteristics of *A. diazotrophicus* include its ability to grow on high sucrose concentration (up to 30%) and very low pH (as low as 3.0), and its ability to grow aerobically but fix N under microaerobic condition (Gillis et al., 1989; Stephan et al., 1991). Of particular interest is the apparent non-inhibitory effect of nitrate on nitrogen fixation (Stephan et al., 1991). This is not common among known diazotrophic bacteria and implies that *A. diazotrophicus* inside sugarcane can continue to fix N even after the addition of nitrate-containing fertilizers.

The relatively high number of *A. diazotrophicus* isolated from surface-sterilized stems, leaves, and roots of sugarcane, as well as microscopic studies, confirmed the endophytic nature of this nitrogen fixing bacterium (Cavalcante and Döbereiner, 1988; James et al., 1994; Dong et al., 1994). *A. diazotrophicus* has not been isolated from non-rhizosphere soil nor from other grasses growing with sugarcane in the same field (Li and MacRae, 1992).

Since its isolation from several sugarcane growing regions of Brazil, A. *diazotrophicus* has also been found in sugarcane growing in Australia, Cuba, Argentina and Mexico (Li and MacRae, 1991; Fuentes-Ramirez et al., 1993; Dong et al., 1994; Bellone et al., 1997). *A. diazotrophicus* has also been isolated from three other plants, Cameroon grass (Pennisetum purpureum), sweet potato (*Ipomeae batatas*) and more recently, coffee (Coffea arabica) (Cavalcante and Döbereiner, 1988; Paula et al., 1991; Jimenez-Salgado et al., 1997). These observations suggest that the association of *A. diazotrophicus* with sugarcane

and other plants may represent a more widespread association than what was initially thought.

The correlation between the presence of this bacterium inside sugarcane grown for years with low N input and ^{15}N-isotope dilution experiments suggest that *A. diazotrophicus* might contribute significant fixed N for plant growth (Urquiaga et al., 1992). However, since other diazotrophs have been isolated from sugarcane (Döbereiner et al., 1988) and there may be others that are unculturable, it is still not certain whether *A. diazotrophicus* is truly responsible for the observed biological fixation in sugarcane. It must also be established whether *A. diazotrophicus* directly benefits plants by transferring fixed N or if other growth-promoting factors are involved.

The focus of our research is to determine the contributions of *A. diazotrophicus* to sugarcane growth and nutrition. Our approach includes the isolation of bacterial genes responsible for nitrogen fixation and their analysis. This strategy led to the isolation of the nitrogenase structural genes, *nifHDK*. Subsequent mutagenesis of *nifD* resulted in the construction of a mutant strain of *A. diazotrophicus* that lost its ability to fix N (Nif$^-$). In preliminary experiments, young plants grew better in N-free medium after inoculation with the wild type strain than with the Nif$^-$ mutant (or uninoculated plants).

2. Materials and Methods

Preparation of A. diazotrophicus genomic library

A. diazotrophicus strain PAl5 was grown as described by Cavalcante and Döbereiner (1988). The CTAB method was used to extract the chromosomal DNA of *A. diazotrophicus* (Ausubel et al., 1992). Standard cloning, DNA manipulations, and preparation of sucrose gradients were as described in Sambrook et al. (1989). Genomic library was prepared in a lambda cloning vector, EMBL3, to allow for the preparation large amounts of DNA to be analyzed in Southern blots.

Isolation and sequencing of nifHDK

The *nifHDK* genes of *A. diazotrophicus* were detected in the EMBL3 genomic library by hybridization with the *nifHDK* region of *Azospirillum brasilense*. Subclones and deletion derivatives of the initial hybridizing clone were sequenced using the dideoxynucleotides method with ^{35}S-dATP. The sequence obtained was deposited in GenBank with the accession number AF030414. The complete nucleotide and the deduced amino acid sequence of *nifHDK* were analyzed using GCG Wisconsin Sequence Analysis Package, v. 8.

Construction of Nif⁻ mutants

A *nifD*:Kan *uidA* (GUS) insertion mutation was constructed by insertion of the Kan *uidA* cassette within the *nifD* region cloned in a suicide vector, pSUP203, which is unable to replicate in *A. diazotrophicus*. Transconjugants in which the *nifD* mutation replaced the wild type gene were isolated. Two of such transconjugants were MAd2B and MAd3A. The ability of these transconjugants to fix N was tested by growing in N-free semi-solid media LGI (Cavalcante and Döbereiner, 1988) and the acetylene reduction assay. Southern hybridization was used to confirm the replacement of wild type genes in these mutants.

Inoculation of sterile sugarcane plants

Micropropagated sterile sugarcane plants of cultivar SP70-1143 were obtained by meristem tissue culture (Hendre et al., 1983; James Irvine, personal communication). Inoculation was done using the method of James et al. (1994) with some modifications (Veronica Reis, personal communication). Rooted plantlets were separated individually into baby food jars containing MS media (Murashige and Skoog, 1962) with and without N. Bacterial inoculum of wild type and Nif⁻ mutant strain was prepared by harvesting 24 hr old bacteria and resuspending in LGI salt solution to an OD_{600} = 0.6. Mutant or wild type bacteria (100 μl) were added to the media. Control plants received 100 μl of LGI salt solution. Inoculated plants were kept on growth shelves at 28°C with 12 hr light and dark diurnal cycle. Ten days after inoculation, five plants from each treatment were examined for colonization by SEM and TEM and by Most Probable Number (MPN). The remaining plants were transferred to sterile sand in conical containers sterilized by bleach. These plants were incubated in a growth chamber under the same conditions as those on growth shelves. Plants were watered with MS medium once every two weeks and with distilled water as needed. Thirty days after inoculation, plant height data and MPN counts were taken.

3. Results and Discussion

Isolation and sequencing of nifHDK

The *A. diazotrophicus nifHDK* gene products are highly similar to those of other nitrogen-fixing bacteria, particularly with other diazotrophic members of the α-subgroup of Proteobacteria. Major structural features common to all known NifHDK proteins are also found in *A. diazotrophicus* NifHDK. As an

Ad	S N V K P V P G U M T I R G C A Y A G S K G V V W
Av	S N K K S Q P G L M T I R G C A Y A G S K G V V W
Kp	S N R K S Q P G V M T V R G C A Y A G S K G V V F
Bj	S N I K S I P G V M T I R G C A Y A G S K G V V W
An	S N I K S V P G V M T A R G C A Y A G S K G V V W
Hs	S N I K S I P G V M T I P P C A Y A G S K G V V W
Ab	S N I K S I P G V M T I R G C A Y A G S K G V V W

Figure 1. Alignment of portion of NifD showing conserved motifs (boxed). Ad, *Acetobacter diazotrophicus*; Av, *Azotobacter vinelandii*; Kp, *Klebsiella pneumoniae*; Bj, *Bradyrhizobium japonicum*; An, *Anabaena* sp.; Hs, *Herbaspirillum seropedicae*; Ab, *Azospirillum brasilense*.

example, an alignment of a portion of the amino acid sequence of *A. diazotrophicus* NifD with that of other diazotrophs is shown in Fig. 1. The indicated conserved motifs are proposed to be significant in the formation of P clusters (Dean and Jacobson, 1992). Conserved motifs in NifH and NifK are also present in the *A. diazotrophicus* proteins (Sevilla et al., 1997; Sevilla et al., manuscript in preparation). The sequences are most similar to those from *R. leguminosarum* bv. *phaseoli* (NifH: 91% identity); *Herbaspirillum seropedicae* and *A. brasilense* (NifD: 91 and 89% identity, respectively), and *Bradyrhizobium japonicum* (NifK: 76% identity). A dendrogram created by GCG PILEUP based on the NifD sequences is shown in Fig. 2. The separation of the archaebacterium *Methanococcus thermolithotrophicus* and the thallobacter *Frankia* from the cluster of the Proteobacteria is apparent in this dendrogram. Overall, the clustering pattern is similar to that obtained from 16S rRNA analysis, with certain exceptions (Young, 1992). Interestingly, the cyanobacterium *Anabaena* NifD clusters more with the Proteobacteria, and the NifD of two members of the β-subgroup, *H. seropedicae* and *Thiobacillus ferrooxidans* are grouped among the rest of the α Proteobacteria. Similar results were obtained by Normand et al. (1992) in their analysis of *Frankia nifD* and other published NifD sequences. The high percent identity of *A. diazotrophicus* and *H. seropedicae* NifD is interesting because *H. seropedicae* is also a diazotrophic endophyte of sugarcane. This may indicate that horizontal transfer of genes is significant between bacteria in confined habitats like the vascular system of plants.

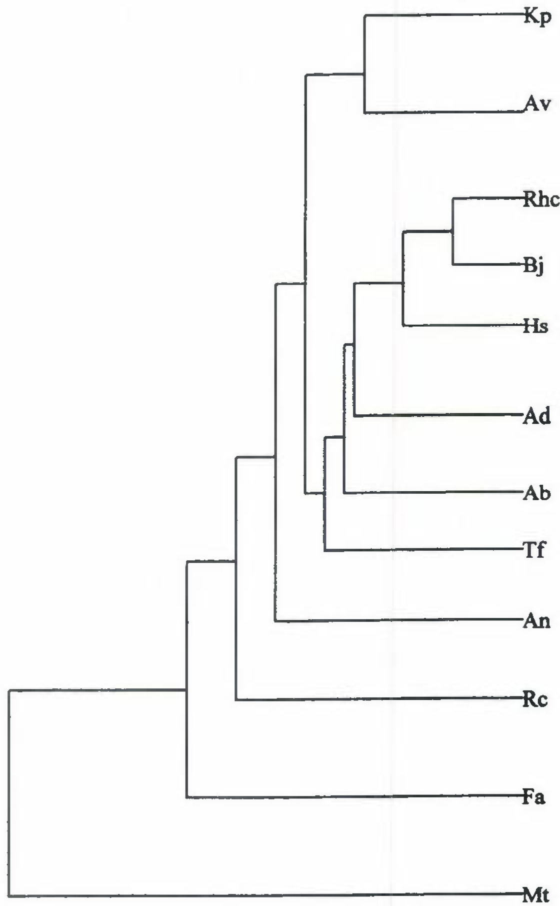

Figure 2. Dendrogram created by PILEUP based on NifD showing the relationship of A. diazotrophicus with other nitrogen fixing bacteria. Kp, *K. pneumoniae*; Av, *A. vinelandii*; Rhc, *Rhizobium* cowpea isolate; Bj, *B. japonicum*; Hs, *H. seropedicae*; Ad, *A. diazotrophicus*; Ab, *A. brasilense*; Tf, *Thiobacillus ferrooxidans*; An, *Anabaena* sp., Rc, *Rhodobacter capsulatus*; Fa, *Frankia* sp. strain Facl; Mt, *Methanococcus thermolithotrophicus*.

Construction of Nif⁻ mutants

Nif⁻ kanamycin resistant transconjugants were unable to grow in N-free media and failed to reduce acetylene to ethylene. Southern analysis confirmed that the *nifD*:Kan *uidA* mutation replaced the wild type *nifD* gene in these mutant strains (data not shown). The Nif⁻ mutants were similar to the wild type in all other phenotypes.

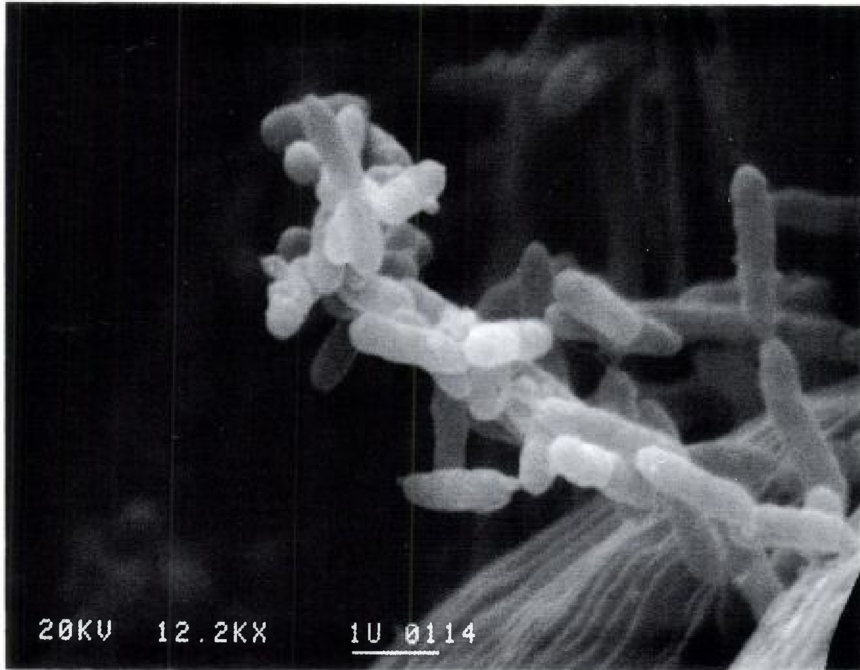

Figure 3. SEM of *A. diazotrophicus* cells inside sugarcane stems, 10 days after inoculation. Note the characteristic clumping of cells (observed in both wild type and Nif^{-} mutant strains).

Inoculation of sterile sugarcane plants

Preliminary experiments showed that micropropagated plants are colonized equally well by wild type and mutant *A. diazotrophicus* whether they are grown in medium with or without N. Scanning electron microscopy and Most Probable Number counting of bacteria from plant homogenates revealed similar numbers of both wild type and mutant strains inside the plant 10 days after inoculation. Bacteria were present in the roots, stems and shoots of inoculated plants. High concentrations of bacteria were observed in the intercellular spaces of tissues examined. Bacterial cells were also observed in the xylem vessels. Bacteria inside plant tissues tended to clump and appear to be held together by a mucilage-like material (Fig. 3). This pattern of colonization is observed for both wild type and Nif^{-} mutants. Similar observations have been reported by James et al. (1994). The significance of bacterial clumping and the mucilaginous material in colonization and nitrogen fixation are currently unknown. It is possible that this arrangement provides some oxygen protection to nitrogen fixing bacterial cells.

The bacteria isolated from inoculated plants were tested by growing in LGI media with 10% sucrose acidified to pH 4.5 with or without kanamycin and by their ability or inability to grow in N-free media and reduce acetylene. In addition, *A. diazotrophicus* specific primers were used in PCR reactions to amplify the 23S rRNA of isolated bacteria (Reis et al., 1995). All yielded an amplification product of about 0.4 kb characteristic of the original *A. diazotrophicus* strain PAl5 used in this study (data not shown). No bacteria were observed in or isolated from uninoculated control plants.

Thirty days after inoculation, heights of inoculated plants were measured (Table 1). The data show that in N-deficient conditions, wild type inoculated plants were taller than both uninoculated and mutant-inoculated plants. The numbers of wild type and mutant bacteria isolated from plant tissues were similar indicating that the plant growth effect is not due to differences in the number of surviving bacteria inside the plant but rather to the fixed N supplied by the wild type *A. diazotrophicus* to sugarcane. The small difference between the two NifD$^-$ mutants tested in the number of bacteria detected is not reflected in a difference in plant height. The variation in cell number may be due to the inefficiency of the homogenization process, and variation in the degree of clumping together of bacteria inside sugarcane tissues. The MPN counting technique is likely to underestimate the true number of endophytic bacterial cells.

Table 1. Average height of sugarcane plants 30 DAI and *A. diazotrophicus* cells/gram tissue (MPN)

Treatments[1]	Height (cm)[2]	Number of cells
0 – N	11.7 d	0
PAl5 – N	16.3 c	5.48×10^5
MAd2B – N	12.7 d	1.67×10^5
MAd3A – N	12.0 d	6.16×10^5
0 + N	18.0 b	0
PAl5 + N	22.6 a	10.5×10^5
MAd2B + N	22.4 a	11.3×10^5
MAd3A + N	21.5 ab	5.70×10^5

[1]N = no nitrogen, +N = with nitrogen (supplied as 4 mM NH_4NO_3 + KNO_3), 0 = not inoculated, PAl5 = wild type, MAd2B = *nifD*$^-$ mutant, MAd3A = *nifD*$^-$ mutant. [2]Tested for significant differences by analysis of variance with Tukey test for multiple comparison, $\alpha = 0.05$.

When N was not limiting, there was no significant difference observed in the heights of wild type- and mutant- inoculated plants. All inoculated plants were significantly taller than the uninoculated control plants. This interesting result suggests the possibility of other beneficial effects of *A. diazotrophicus* to sugarcane growth in addition to supplying fixed nitrogen. That growth promoting substances are also involved is likely since *A. diazotrophicus* has been shown to produce significant amounts of indole acetic acid (Fuentes-Ramirez et al., 1993). Strain PAl5 was found to be the highest IAA producer among the ten *A. diazotrophicus* strains examined.

More plant experiments are underway to follow the inoculated young plants beyond 30 days after inoculation. Other parameters such as shoot and root dry weight, as well as plant tissue N content analysis will be measured. It will also be important to carry out N balance and ^{15}N dilution experiments to ascertain the amount of contributed N provided by *A. diazotrophicus* to sugarcane. Such experiments will help to establish whether the association between *A. diazotrophicus* and sugarcane represents a new type of true symbiosis.

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