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# A diazotrophic bacterial endophyte isolated from stems of *Zea mays* L. and *Zea luxurians* Iltis and Doebley

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## Abstract

The apoplastic fluids of field-grown *Zea mays* and *Zea luxurians* plants were isolated from surface sterilized stem tissue by centrifugation and spread on agar plates containing a nitrogen-free, defined medium. The predominant bacterium isolated from these plates was characterized further. The ability of this bacterium to fix nitrogen was confirmed by its ability to grow on a semi-solid, nitrogen-free medium and reduce $^{15}N_2$ to $^{15}NH_3$ and acetylene to ethylene. Portions of the *nifH* and 16S rRNA genes from this organism were amplified by PCR and sequenced. The *nifH* gene, which codes for dinitrogenase reductase, from this organism is closely related to *nifH* from *Klebsiella pneumoniae.* Similarly, the 16S rRNA gene sequences and carbon utilization tests grouped it closely with *K. pneumoniae.* Based an these data, the isolates from *Z. mays* and *Z. luxurians* are tentatively classified as *Klebsiella* spp. (*Zea*). The ability of this bacterium to contribute to the nitrogen economy of the corn plant is unknown.

## Introduction

Biologists at EMBRAPA in Rio de Janiero have found large numbers of the bacterium *Acetobacter diazotrophicus* in the stem tissue of certain cultivars of sugarcane (Boddey et al., 1991; Reis et al., 1990). *A. diazotrophicus* is believed to fix nitrogen in the stem tissue of sugarcane using the large amount of sucrose present as a carbon source. Certain cultivars of sugarcane, not fertilized with nitrogen but infected with *Acetobacter*, do not require the addition of any fertilizer nitrogen to achieve optimum yields (Boddey et al., 1991; Urquiaga et al., 1992). However, no one has made the appropriate *nif* mutants to demonstrate that *A. diazotrophicus* is a source of fixed nitrogen for sugarcane. *Acetobacter* has also been shown to be an endophyte of sugarcane cultivars in Australia, Cuba, and Mexico (Dong et al., 1994; Fuentes-Ramirez et al., 1993; Li and MacRae, 1992).

*Acetobacter* enters sugarcane through mycorrhizae (Boddey et al., 1991; Paula et al., 1991; Reis et al., 1990). Once established in the stem, the endophyte is spread by vegetative propagation, the primary method of sugarcane establishment.

Our primary objective is to determine whether a similar diazotrophic association can be demonstrated or developed for sweet corn. In support of this notion, Fisher et al. (1992) have found that healthy maize plants in England possess endophytic bacteria and fungi. The most common bacterial endophytes in maize were identified using the Biolog system as strains of *Enterobacter agglomerans, Klebsiella terrigena, Pseudomonas corrugata, P. fluorescens, P. marginalis, P.* sp., 4, *P.* sp. 5, and *Vibrio* sp. 1. Strains of *Vibrio, Pseudomonas, E. agglomerans* and *K. terrigena* have been shown to be diazotrophs (for review see Young, 1992). Thus, it seems likely that diazotrophs can inhabit maize. However, the ability of the Biolog system to correctly identify strains of *Enterobacter* and *Klebsiella* is in doubt (Klingler et al., 1992).

McInroy and Kloepper (1991, 1995) have also isolated and provided preliminary identification for bacterial endophytes of maize. These workers found 29 genera of bacteria in field-grown stems and roots of maize. Among these genera were several that include diazotrophic members such as *Bacillus, Enterobacter,*

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*Erwinia, Klebsiella,* and *Xanthobacter* (Young, 1992). The bacteria in this work were identified by gas chromatographic analysis of fatty acid methyl esters.

McInroy and Kloepper (1991, 1995) have also made four other interesting observations. First, the number of bacterial genera found in maize stems decreased with the age of the plant. At plant maturity, *Pseudomonas* and *Bacillus* strains were dominant. Second, maize tissue was found to contain about $10^4$ bacteria per gram fresh weight of tissue. Third, surface-sterilized seeds possessed $10^5$ to $10^6$ bacteria per gram fresh weight. Fourth, plants grown under sterile conditions from surface-sterilized seeds also contained stem bacteria but at levels 100-fold lower than plants cultured in non-sterile soil. This suggests that the endophytic bacteria can be transmitted either through seed or soil. The observation of seed transmission is potentially important for annual species such as maize and suggests a simple method of inoculation of plants via seed coating or infiltration.

No one to date has shown whether any bacterial endophytes of maize are capable of nitrogen fixation either in vitro or in planta. The objective of the work described here was to determine whether a diazotroph could be isolated from field grown maize from Wisconsin.

## Materials and methods

### *Source of plants and harvesting of tissue*

Mature sweet corn, *Zea mays* L. cv. Excellency, and teosinte, *Z. luxurians* Iltis and Doebley, plants were harvested from the University of Wisconsin West Madison Farm in 1994. The sweet corn and teosinte were harvested at sites located at 43°03.68N, 89°32.54W and 43°03.76N, 89°32.54W, respectively. Mature plants of each species was cut near the base, placed in water, and cut under water to limit air from entering the stem.

### *Media used for bacterial isolation and culture*

Isolation of diazotrophic bacteria was done using a modified LGI medium prepared as described by Cavalcante and Döbereiner (1988) except that 10% sucrose replaced crystalized cane sugar and the yeast extract was omitted. One an one-half percent agar was added for solid media and 0.05% agar was added for semisolid media (sLGI). Tryptic Soy Agar (TSA, Difco) was used to increase cell numbers rapidly. For determination of nitrogenase activity, the bacteria were cultured in AcD medium developed by R H Burris (unpublished), which includes (per liter): 3 g $K_2HPO_4 \cdot 3H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaSO_4 \cdot 2H_2O$, 0.2 g NaCl, 5 mg $(NH_4)_2SO_4$, 3 mg $FeCl_3$, 1 mg $Na_2MoO_4 \cdot 2H_2O$, 0.1 g yeast extract, and 20 g sucrose adjusted to pH 6.5 with 85% $H_3PO_4$.

### *Surface sterilization of stem tissue and culture of bacteria*

A section of stem, roughly six cm in length, was cut near the base of the mature plants, from which the leaf was removed and the section was immersed in 70% ethanol and flamed as described by Dong et al. (1994). Using a sterile scalpel, two cm of stem cross section was removed from the sterilized stem. This step, in addition to the removal of the surface leaf tissue were done in order to avoid inadvertent contamination of bacterial surface inhabitants in our apoplastic fluid samples. Apoplastic fluid from each maize stem was removed by centrifuging stem sections at 3000 *g* for 20 min as described by Dong et al. (1994). Apoplastic fluid, 0.3 mL, was recovered from each, diluted 1:1 with 30% glycerol and stored at -75 °C. The apoplastic fluid from each maize stem was serially diluted, plated on LGI, and incubated at 28 °C for up to five days. Nineteen separate colonies were kept and cleaned on LGI from the mature maize, cultured thickly on TSA and stored in 15% glycerol at -75 °C.

All 19 isolates from the mature maize were inoculated with a needle into 5 mL sLGI and incubated at 28 °C. Cultures were reinoculated into fresh sLGI after one week and this transfer was repeated twice. Seven of the 19 isolates grew visibly during the final incubation. These seven isolates were identical with respect to colony morphology, cell shape and size, and antibiotic sensitivity.

### *PCR amplification and sequencing of partial 16S rRNA and nifH genes*

Genomic DNA was extracted from the isolates by the method of Sambrook et al. (1989) and the 16S rRNA gene was amplified from four isolates, three taken from *Z. mays* and one taken from *Z. luxurians,* and *K. pneumoniae* URI with PCR in an air thermocycler. The oligonucleotide primer sequences (Angert et al., 1993) used were TR7 (5′-GCTGCAGAGTTTGATCCTGGCTCAG-3′) and

TR8 (5′-CGGGTTACCTTGTTACGACTT-3′) with reactants as described by Wittwer (1992) using the following conditions: denaturation at 92 °C for one minute; 35 cycles of denaturation at 92 °C for 10s, annealing at 50 °C for 30s, and elongation at 72 °C for 60s; and a final elongation at 72 °C for three minutes. PCR products were purified using the Wizard DNA purification kit (Promega).

PCR products were sequenced using ABI's (Perkin-Elmer) dideoxy terminator cycle sequencing kit. Sequence analysis was done using an automated ABI sequencer at the University of Florida Interdisciplinary Center for Biotechnclogy Research or at the University of Wisconsin-Madison Department of Horticulture. Primers used for the sequencing reactions were TR7, TR8, and six additional primers: 16SF2 (5′-CTGAGACACGGCCCAGACTC-3′), 16SF3 (5′-GCGTAGAGATCTGGAGGAATA-3′), SR2R (5′-TCCCGCAACGAGCGCAACCC-3′), 16SR2 (5′-GGGTTGCGCTCGTTGCGGGA-3′), 16SR3 (5′-TTCGCCACCGGTATTCCTCCA-3′), and SF2R (5′-GAGTCTGGGCCGTGTCTCAG-3′).

A partial *nifH* sequence of one isolate was amplified with PGR using universal primers described by Ueda et al. (1995) and later with primers based on *K. pneumonia* sequence nifh.f (5′-ACCATGCGTCAATGCGCATT-3′) and nifh.r (5′-TCGGTCTGACGTGAGTTACA-3′). Reaction conditions were 94 °C for one minute; 35 cycles at 94 °C for 10s, 55 °C for 30s, and 75 °C for 60s; and a final elongation of 75 °C for three minutes. Processing of the PCR products was identical to that for the 16S rDNA and primers used for sequencing were the universal forward and the Nifh.f primers. All sequences were entered in the GenBank database and given accession numbers.

*Phylogenetic analysis of the nifH and 16S rRNA genes*

All sequence analyses were performed using programs of the Genetics Computer Group Inc. (Madison, WI) unless otherwise stated. Sequences of 16S rDNA with high homology to the new sequences described here were identified using the BLAST program. The *nifH* sequences used were compiled by Ueda et al. (1995). These sequences were then aligned using the PILE-UP program. Using MEGA (Kumar et al., 1993) the aligned sequences were constructed into phylogenetic trees with a pairwise distance matrix, using the Jukes-Cantor algorithm and the Neighbor-Joining method.

*Acetylene and $^{15}N_2$ reduction*

One bacterium that grew in sLGI was inoculated into 50 ml of AcD and cultured without shaking for 36 hours. Five milliliters of culture were added to each of six 38 mL serum bottles. Three milliliters acetylene was added and samples (1.0 mL) were removed after incubation for zero, four, and eight hours. The amount of ethylene in a 0.5 mL sample was measured by using a Shimadzu gas chromotograph. Reduction of $^{15}N_2$ to $^{15}NH_3$ was as described by Burris (1972) using 5 mL cultures of cells cultured in AcD medium in a 30 mL serum vial. Total cell protein was determined by a modified Lowry procedure (1977) using bovine serum albumin as a standard.

*Physiological characteristics of isolates*

Many physiological characteristics of the maize endophytes were determined using the API 20E kit (bioMÉRIEUX VITEK, Hazelwood, MO).

*Quantification of the number of diaztrophic isolates in maize tissue*

The apoplastic fluids from *Z. mays* and *Z. luxurians* were diluted appropriately to observe single colonies and the dilutions spread on the modified LGI medium. The number of colonies which grew on this medium were counted. About thirty of these colonies were then inoculated into semi-solid modified LGI medium to determine the number of these that could grow in the N-free medium. Based on the proportion of colonies that grew in the semisolid medium compared to the number that grew on solid medium and based on the fresh weight of the maize samples used for the isolation of the apoplastic fluids, the number of diazotrophs that grow on the semi-solid modified LGI medium per g fresh weight of maize stem tissue was determined.

## Results

*Strain characterization*

Seven of the 19 isolates kept from the apoplastic fluids of *Zea mays* and *Z. luxurians* grew in semi-solid LGI. These seven isolates had identical phenotypes with respect to the following characteristics: a) small orange colony morphology on LGI supplemented with 1 g $L^{-1}$ $NH_4Cl$; b) large white colony morphology on TSA; c)

rod shape when simple stained with gram violet and viewed at 1000×; d) acidification of Kligler's Iron Agar; e) resistance to 25 $\mu g\ mL^{-1}$ penicillin, carbenicillin, rifamycin, ampicillin, erythromycin, cyclohexamide, trimethoprin, and fusaric acid; f) sensitivity to 10 $\mu g\ mL^{-1}$ gentamycin, spectinomycin, kanamycin, naladixic acid, tetracycline, streptomycin, and chloramphenicol; and h) growth on TSA with 5% NaCl. Two *Z. mays* isolates were analyzed in detail and are referred to as strains zmmo and zmvsy. One *Z. luxurians* isolate was also examined and is referred to as strain zlmy.

*Physiological characteristics of the maize isolates*

Seventeen physiological characteristics of zmvsy and zlmy were determined and were found to be identical in all cases to each other and to *Klebsiella pneumoniae*. The maize isolates were capable of utilizing all carbon sources tested including citrate, glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, and arabinose. They also tested positive for ONPG hydrolysis, lysine decarboxylation, and urease production; tests for arginine dihydrolase and production of $H_2S$, indole, and acetoin were negative. They did not liquify gelatin.

*Quantification of the number of diazotrophic isolates in maize tissue*

The number of colonies capable of growing on semi-solid N-free medium was found to be $2.2 \times 10^3$ and $1.4 \times 10^1$ per gram of fresh weight in *Z. mays* and *Z. luxurians*, respectively. The colony morphology of the cells which grew in this medium were very similar to each other suggesting that they are the same as, or very similar to, strains zmmo, zmvsy, and zlmy. The number of these diazotrophic bacteria found in *Zea mays*, $2.2 \times 10^3$, is at the lower limit of the amount found in sugarcane (Dobereiner et al., 1988). The number found in *Z. luxurians* is considerably lower.

*Sequence analysis of 16S rRNA genes*

As the isolates obtained from the N-free medium were identical by the above criteria, three of the strains, zmmo, and zmvsy from *Zea mays* and zlmy from *Zea luxurians*, were characterized in more detail. These three isolates had 16S rRNA gene sequences which differed in only one or three bases among ca. 1500 bp sequenced. The nearly complete sequences of the 16S rRNA gene from these isolates were also compared with those of other 16S rDNA sequences in the database (Figure 1). The sequences reported in this paper have been deposited in the GenBank data base (16S accession numbers: zmvsy, U31076; zmmo, U31075; zlmy, U32868; *K. pneumoniae*, U33121)

*Sequence analysis of nifH*

The partial *nifH* sequence from zmvsy was amplified and sequenced. In comparison with other *nifH* sequences in the database, the zmvsy *nifH* sequence was most similar to that of *Klebsiella pneumoniae* with 84.5% identity over 251 bp, (Figure 2). The *nifH* accession number for zmvsy is U32183).

*Nitrogenase activity*

The nitrogenase activity of zmvsy was determined by acetylene reduction and $^{15}N_2$ reduction. The acetylene reduction rate of zmvsy under the conditions described above was 1.1 $\mu$mol ethylene produced per hr per mg protein. This strain assimilated 4.04% of its total nitrogen in 220 minutes as determined by $^{15}N_2$ reduction.

## Discussion

This work demonstrates that the stems of *Zea* spp. are capable of maintaining a population of diazotraphic bacteria. The diazotrophic nature of these isolates was shown by the reduction of $^{15}N_2$ and acetylene and by their ability to grow well in a semi-solid nitrogen-free medium.

Under the conditions used here for the isolation of diazotrophs, only strains with nearly identical characteristics were identified. Given that corn and sugarcane are closely related, we expected to find diazotrophs that were similar to those found in sugarcane, such as *A. diazotrophicus*. For that reason, we chose a medium for diazotroph isolation that was used by Dong et al. (1994) in the isolation of *Acetobacter* from sugarcane. No strains similar to *Acetobacter* were found in maize stems in this work. Instead, a new diazotroph was discovered with characteristics very similar to *Klebsiella*.

Several lines of evidence show that zmmo, zmvsy, and zlmy are in the $\gamma$-subdivision of the proteobacteria. The *nifH* and 16S rDNA sequence data both show that these isolates from maize are enterics which are close relatives of *Klebsiella, Serratia, Citrobacter* and *Erwinia*. The isolates also acidified Kligler's iron agar and grew on a medium containing a high salt concen-

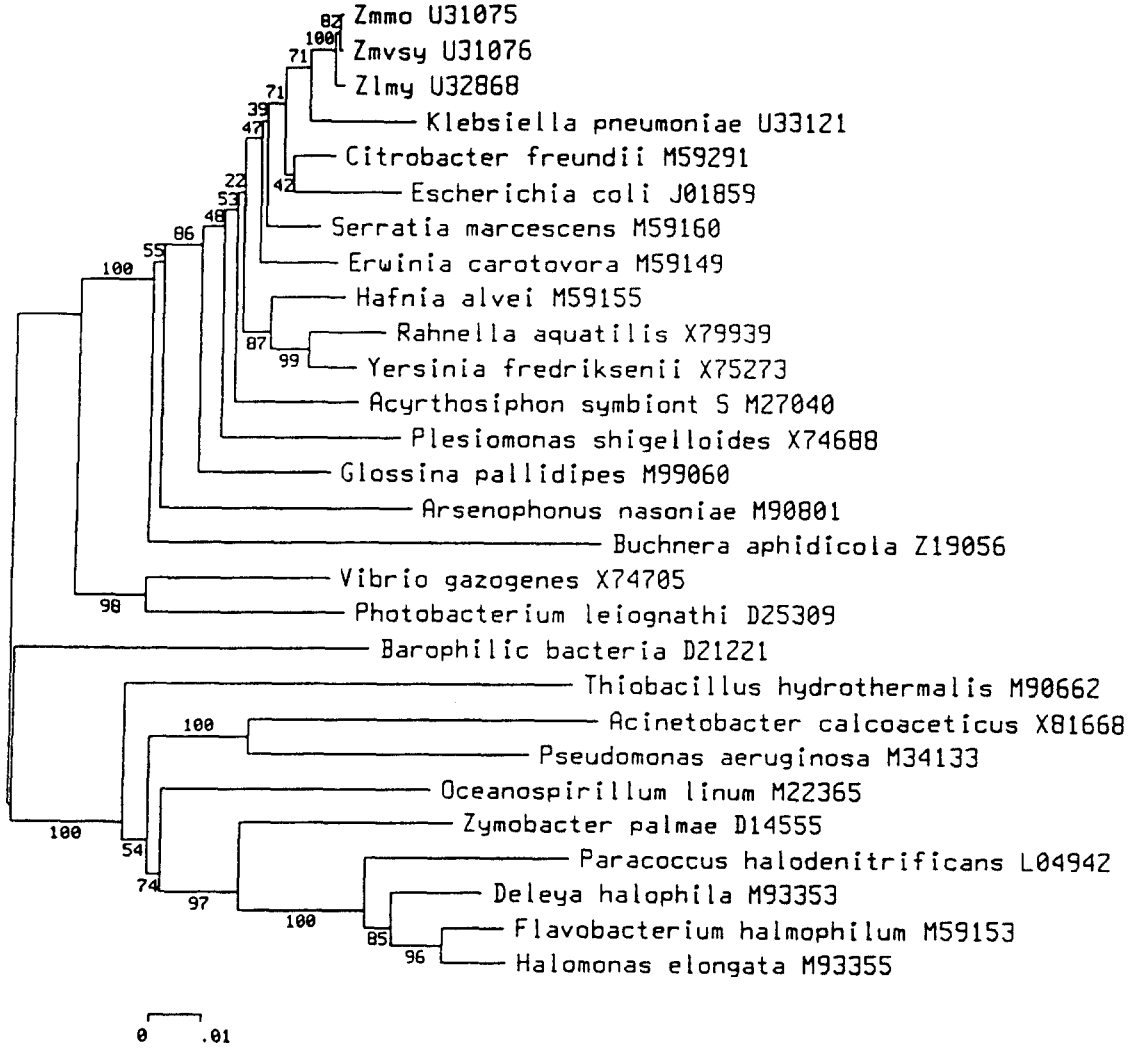


*Figure 1.* Phylogenetic tree of 16S rDNA sequences of the *Klebsiella* spp. (*Zea*) isolates and selected bacteria from the GenBank database. Confidence levels (%) above each node were generated from 500 bootstrap trees. The distance scale represents the number of changes per sequence position with a median rate of change.

tration, both qualities supporting their enteric nature. Although more work is necessary to propose a taxon for these strains, their close relationship to *K. pneumoniae,* as illustrated by 16S rDNA sequence comparisons and by physiological characteristics, supports the notion of giving these strains the preliminary designation *Klebsiella* spp. (*Zea*).

The development of nitrogen-fixing corn has often been touted as one of the ultimate goals of nitrogen fixation research. Several approaches have been described in the past for the development of nitrogen-fixing maize. These include: a) the transfer of nodulation genes from a legume to maize for the purpose of inducing nitrogen-fixing nodules on maize (Dixon et al., 1993); b) the expression of the bacterial *nif* regulon in maize organelles ( Dixon et al., 1993); and c) the development of maize lines with the ability to accept fixed nitrogen from diazotrophs in the rhizophere (Ela et al., 1982). All of these proposals have enormous technical problems to overcome such that the development of nitrogen-fixing maize in the near term has been considered unlikely.

However, by adapting the sugarcane paradigm for a similar strategy for maize, nitrogen-fixing maize may be possible without large technical impediments. This paper represents the first step in this strategy which is the identification of endophytic diazotrophs in maize.

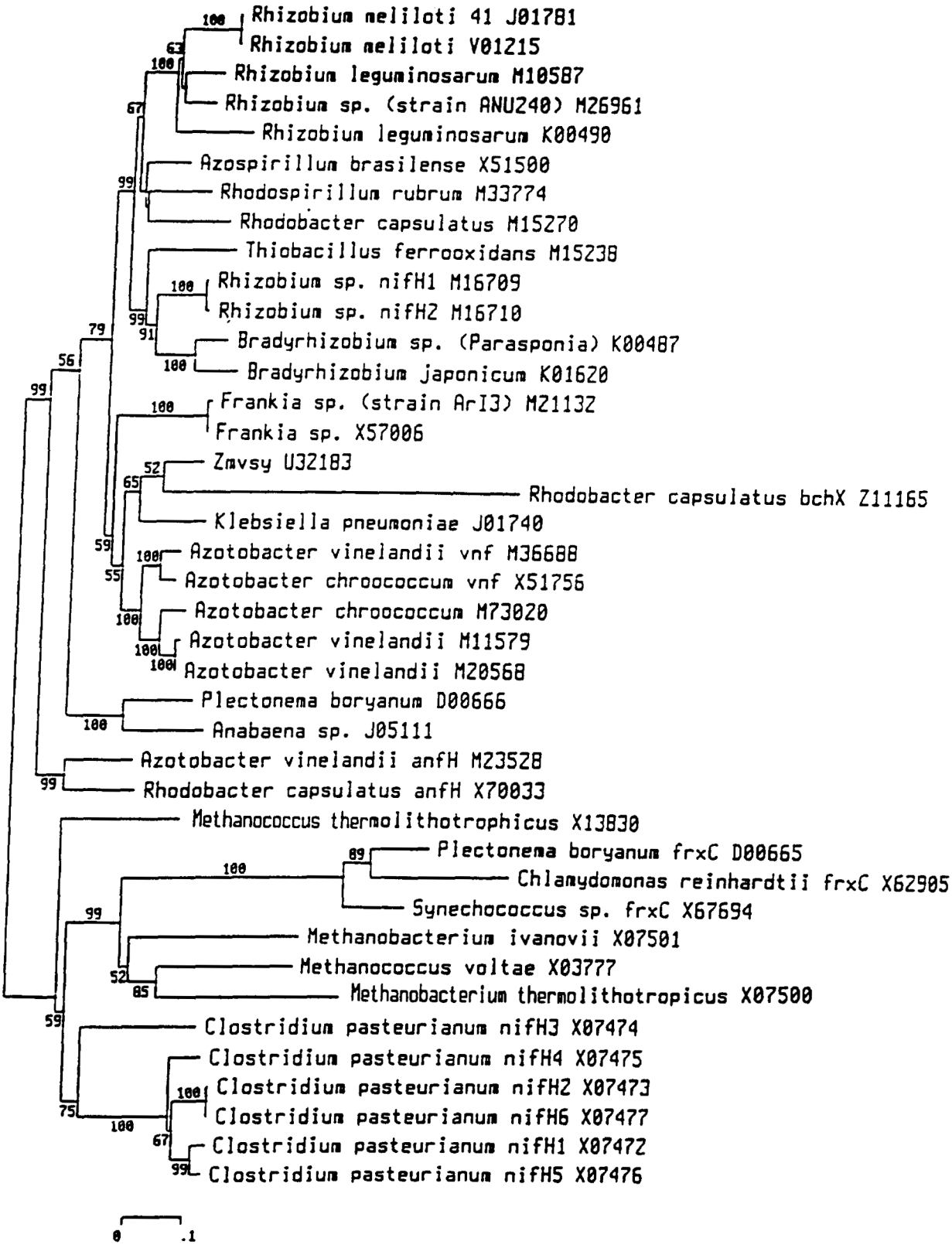


*Figure 2.* Phlogenetic tree of selected *nifH* sequences (Ueda et al., 1995) and *Klebsiella* spp. (*Zea*) strain zmvs. Confidence levels (%) above each node were generated from 500 bootstrap trees. The distance scale represents the number of changes per sequence position with a median rate of change.

The mechanism by which these bacteria enter the plant and the ability of these strains to provide any benefit to the nitrogen economy of maize is unknown. The plants chosen for bacterial isolation were cultured under conditions of high nitrogen fertilization so it is reasonable to assume that the nitrogenase system of these endophytes was repressed. The apoplast is likely to contain that nitrogen source, most likely nitrate, absorbed by the plant from the soil.

*Herbaspirillum seropedicae* is a diazotroph which has also been described as a maize endophyte. To date, it has been found in roots only (Baldani et al., 1986, 1992; Döbereiner et al., 1993). *Herbaspirillum seropedicae* is pathogenic on *Sorghum* and *Pennisetum* (Döbereiner et al., 1993; Pimentel et al., 1991). Thus, its exploitation as a potential inoculant for corn is doubtful. Also, in contrast to the *Klebsiella* spp. (*Zea*) strains described here, *H. seropedicae* cannot use sucrose as a carbon source (Baldani et al., 1986). Because sucrose is in abundance in corn (Bertolini et al., 1993), the prediction could be made that a more successful diazotroph in corn would be one, such as *Klebsiella* spp. (*Zea*), that thrives on sucrose as a carbon source.

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