

# Endophytic *nifH* gene diversity in African sweet potato

Birgit Reiter, Helmut Bürgmann, Kornel Burg, and Angela Sessitsch

**Abstract:** A cultivation-independent approach was used to identify potentially nitrogen-fixing endophytes in seven sweet potato varieties collected in Uganda and Kenya. Nitrogenase reductase genes (*nifH*) were amplified by PCR, and amplicons were cloned in *Escherichia coli*. Clones were grouped by restriction fragment length polymorphism analysis, and representative *nifH* genes were sequenced. The resulting sequences had high homologies to nitrogenase reductases from  $\alpha$ -,  $\beta$ -, and  $\gamma$ -*Proteobacteria* and low G+C Gram positives, however, about 50% of the sequences derived from rhizobia. Several highly similar or even identical nitrogenase reductase sequences clustering with different bacterial genera and species, including *Sinorhizobium meliloti*, *Rhizobium* sp. NGR234, *Rhizobium etli*, *Klebsiella pneumoniae*, and *Paenibacillus odorifer*, could be detected in different plants grown in distinct geographic locations. This suggests that these bacterial species preferentially colonize African sweet potato as endophytes and that the diazotrophic, endophytic microflora is determined only to a low degree by the plant genotype or the soil microflora.

**Key words:** endophytes, nitrogenase reductase, *nifH*, nitrogen fixation, sweet potato.

**Résumé :** Une approche indépendante de la culture a été employée afin d'identifier des endophytes potentiellement fixateurs d'azote dans sept variétés de patates provenant de l'Uganda et du Kenya. Les gènes de la nitrogénase réductase (*nifH*) ont été amplifiés par PCR et les amplicons ont été clonés dans *Escherichia coli*. Les clones ont été regroupés par une analyse du polymorphisme de taille des fragments de restriction et des gènes *nifH* représentatifs ont été séquencés. Les séquences obtenues démontraient de fortes homologies avec les nitrogénase réductases des protéobactéries  $\alpha$ ,  $\beta$  et  $\gamma$  et des gram-positifs à bas G+C. Toutefois, environ 50 % des séquences étaient dérivées de rhizobiums. Plusieurs séquences de nitrogénase réductases hautement semblables ou identiques et se regroupant avec divers genres et espèces de bactéries incluant *Sinorhizobium meliloti*, *Rhizobium* sp. NGR234, *Rhizobium etli*, *Klebsiella pneumoniae* et *Paenibacillus odorifer* ont pu être détectées chez différentes plantes cultivées dans des emplacements géographiques distincts. Ceci indique que ces espèces de bactéries colonisent préférentiellement la patate africaine en tant qu'endophytes, et que la microflore diazotrophique endophyte n'est déterminée qu'à un faible niveau par le génotype de la plante ou par la microflore du sol.

**Mots clés :** endophytes, nitrogénase réductase, *nifH*, fixation de l'azote, patate.

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## Introduction

Sweet potato (*Ipomoea batatas*) is known for its ability to grow well in infertile, nitrogen (N)-poor soils, and over 95% of the global sweet potato crop is produced in developing countries, where it is the fifth most important food crop. Previous reports indicated that associative  $N_2$  fixation contributes to the N uptake (Yoneyama et al. 1998; Hill et al. 1990). Two decades ago Hill et al. (1983) characterized  $N_2$ -fixing *Azospirillum* spp. that were isolated from sweet potato roots. In addition, diazotrophic *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) strains were shown to colonize aerial parts of field-grown sweet potato plants

(Paula et al. 1991). Recently, endophytic diazotrophic bacteria have been isolated from various nonleguminous plants (for reviews see Reis et al. 2000; Reinhold-Hurek and Hurek 1998), including grasses (Baldani et al. 1996; Reinhold et al. 1986; Reinhold-Hurek et al. 1993), coffee (Jimenez-Salgado et al. 1997), and sugarcane (James et al. 1994). Furthermore, a variety of diazotrophs have been isolated from rice (Lahda et al. 1997; Yanni et al. 1997), and an unexpected high diversity of diazotrophs has been found in banana and pineapple (Cruz et al. 2001; Tapia-Hernandez et al. 2000).

Most of the above-mentioned studies used cultivation and subsequent characterization of isolates. However, cultivation of microorganisms can provide only limited information on microbial diversity because of the unculturability of many microorganisms by standard isolation techniques. The application of cultivation-independent methods based on the use of phylogenetic markers, such as the 16S rRNA gene, has improved our knowledge of the endophytic microflora of plants (Garbeva et al. 2001; Sessitsch et al. 2002a; Reiter et al. 2002; Chelius and Triplett 2001). The application of functional genes narrows down the analysis to a functionally defined group of microorganisms. For the description of  $N_2$ -fixing organisms, the *nifH* gene encoding dinitrogenase

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reductase has proved to be a suitable marker, and it has been demonstrated that phylogenetic inferences based on *NifH* amino acid sequences agree with rRNA data (Young 1992). Nitrogen-fixing microbial communities in different environmental systems have been described with the *nifH* gene as phylogenetic marker (Ueda et al. 1995; Ohkuma et al. 1996; Widmer et al. 1999; Kirshstein et al. 1991).

In this study, we analyzed the diversity of potentially diazotrophic bacteria colonizing the interior of sweet potato plants by direct PCR amplification and subsequent cloning and sequence analysis of partial *nifH* genes. Our aim was to identify the major taxonomic groups of bacteria that might contribute to the N input in sweet potato plants grown in different soils on small scale farms in Africa, where no mineral N was applied.

## Materials and methods

### Sample collection

Stem and tuber samples from seven sweet potato cultivars were taken in June 2000 from four small subsistence farms in Uganda (Soroti) and Kenya (Nairobi and Embu), for a total of 14 samples. Soroti is located in eastern Uganda, where the climate is tropical and generally rainy with two dry seasons. Nairobi and Embu lie in the Kenyan highlands, where the climate is mostly arid. The cultivars *I. batatas* 'Araka white' and 'New Araka' were sampled in Soroti, whereas *I. batatas* 'Muibai' and 'Guikanda' were obtained from Nairobi. The cultivars *I. batatas* 'Embu local', 'Embu local 1', and 'Embu local 2' were sampled in Embu.

### DNA isolation

To avoid the isolation of surface bacterial DNA, stems and tubers were soaked in 5% sodium hypochloride (bleach) for 10 min; rinsed four times with sterile, distilled water; rinsed with ethanol; flamed; and peeled aseptically. Stems and tubers were cut into pieces and lyophilized.

For the isolation of DNA, 0.2–0.5 g lyophilized plant tissue were amended with 0.8 mL of TN150 (10 mmol·L<sup>-1</sup> Tris-HCl (pH 8.0); 150 mmol·L<sup>-1</sup> NaCl), frozen in liquid N, and pulverized in a mixer mill (Type MM2000, 220V, 50 Hz, Retsch GmbH & Co KG, Haam, Germany) in the presence of two sterile stainless steel beads (5 mm) at thawing. Then, 0.3 g of 0.1 mm acid-washed glass beads (Sigma) were added, and bead beating was performed twice for 1 min at full speed in a mixer mill. After extracting with phenol and chloroform, DNA was precipitated with 0.1 vol. of 3 mol·L<sup>-1</sup> sodium acetate solution and 0.7 vol. of isopropanol for 20 min at -20 °C. DNA was centrifuged for 10 min at 14 000 r/min (1 r = 2π rad; 10 000g), washed with 70% ethanol, and dried. Finally, the DNA was resuspended in 60 μL of Tris-EDTA buffer containing RNase (0.1 mg·mL<sup>-1</sup>).

### PCR amplification of *nifH* genes

For the amplification of *nifH* gene fragments (371 bp), a nested PCR with degenerate primers was applied (Widmer et al. 1999). Essentially, three primers were used, which were designed to match gene sequences of molybdenum nitrogenase reductase of a broad range of bacteria (Widmer et al. 1999). The first reaction amplified nucleotides 19–482, whereas the nested PCR amplified nucleotides 112–482 of

the *nifH* gene. The same PCR conditions, as described in detail by Widmer et al. (1999), with approximately 100 ng DNA solution as template were used.

### Cloning and RFLP analysis

For cloning, PCR products were purified with the NucleotraPCR kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. DNA fragments were ligated into the pGem-T vector (Promega, Mannheim, Germany) with T4 DNA ligase (Promega), and ligation products were transformed into novablue Singles competent cells (Novagen, Madison, Wis.), as recommended by the manufacturer. Positive recombinants, appearing as white colonies on indicator plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside), were picked and resuspended in 50 μL of PCR reaction mix. Sixteen to 24 clones per plant genotype and tissue (263 in total) were randomly selected and reamplified. PCR products were digested with *AluI* (Invitrogen, Lofer, Austria). One representative of each of the resulting restriction fragment length polymorphism (RFLP) groups of each plant was sequenced.

### Sequence analysis

Cloned *nifH* gene fragments were amplified with the primers M13for and M13rev under previously described PCR conditions (Reiter et al. 2002). PCR products were purified with the NucleotraPCR kit (Macherey-Nagel), according to the manufacturer's instructions and applied as templates in sequencing reactions. Sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977) with the primers M13for and M13rev, respectively, using an ABI 373A automated DNA sequencer and the ABI PRISM Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems Inc., Foster City, Calif., U.S.A.). Nucleotide and amino acid sequences were subjected to BLAST analysis (Altschul et al. 1997) with the National Center for Biotechnology Information (NCBI) database. Sequence alignments were constructed with the Multalin alignment tool (<http://www.toulouse.inra.fr/multalin.html>) (Corpet 1988). Phylogenetic trees of *nifH*-derived amino acid sequences were constructed with the Jukes-Cantor distance estimation and neighbour-joining algorithm using the TREECON software package (van de Peer and de Wachter 1994). The relative abundance of a certain sequence within a plant was defined as the number of clones showing identical RFLP patterns compared with the total number of *nifH* clones analyzed for that plant.

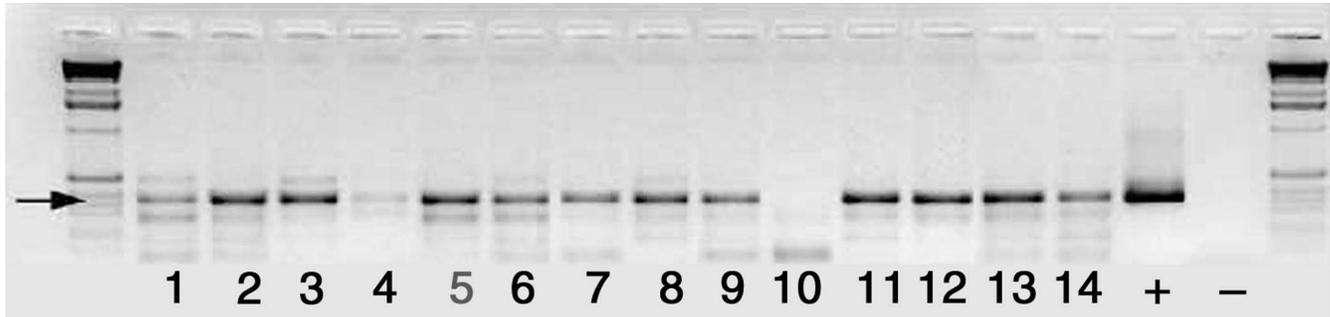
### Nucleotide sequence accession numbers

The sequences determined in this study were deposited in the GenBank database with the accession numbers AY159587-AY159603 and AY303221-AY303228.

## Results and discussion

Amplicons of *nifH* gene fragments were obtained from all plant samples except tubers of the variety 'Embu local 2' (Fig. 1). We identified a total of 17 RFLP groups, and one group representative of each plant was sequenced, resulting in *nifH* sequences. Two of them (*nifH*30 and *nifH*74), al-

**Fig. 1.** Nested PCR amplification of *nifH* genes from sweet potato (*Ipomoea batatas*) stems and tubers. The molecular weight standard (St) was a 100 bp ladder. The sweet potato cultivars were as follows: (1) 'New Araka' stem, (2) 'New Araka' tuber, (3) 'Araka white' stem, (4) 'Araka white' tuber, (5) 'Embu local' stem, (6) 'Embu local 1' root, (7) 'Embu local 1' stem, (8) 'Embu local 1' tuber, (9) 'Embu local 2' stem, (10) 'Embu local 2' tuber, (11) 'Guikanda' stem, (12) 'Guikanda' tuber, (13) 'Muibai' stem, and (14) 'Muibai' tuber. *Azotobacter vinelandii* DNA served as a positive (+) control and for the negative (-) control we used water instead of DNA.



though identical, were found in different sweet potato cultivars, which had been grown in Uganda and Kenya, respectively (Table 1). Eight clones showed at least 96% sequence identity with *nifH* genes deposited in the NCBI database, whereas 11 clones had homologies lower than 93% to known nitrogenase reductase genes. However, at the protein level these clones shared 78%–100% identical amino acids with published NifH sequences (Table 1). These results indicate a high diversity of potentially  $N_2$ -fixing endophytic bacteria in sweet potato, as previously demonstrated in several other investigations analyzing the *nifH* gene diversity in endophyte populations of other plant species (Cruz et al. 2001; Lovell et al. 2001; Ueda et al. 1995). In addition, the clones of four plant samples ('New Araka' stem, 'Araka white' tuber, 'Embu local 1', stem and 'Muibai' stem) contained a sequence of the expected size, but the sequences showed similarity to different hypothetical proteins of *Mesorhizobium loti*. In 'Guikanda' stem and 'Muibai' tuber tissue this sequence was found in addition to *nifH* genes.

Phylogenetic analysis showed that the sweet potato NifH sequences clustered in various taxonomic groups, including  $\alpha$ -,  $\beta$ -, and  $\gamma$ -*Proteobacteria* as well as low G+C Gram positives (Fig. 2). However, about 50% of all nitrogenase reductase genes derived from rhizobia or relatives belonging to the  $\alpha$ -*Proteobacteria*, which are able to nodulate and to fix N with mostly leguminous plants. Amino acid sequences showed highest similarity to four rhizobial species, *Sinorhizobium meliloti*, *Rhizobium etli*, *Bradyrhizobium* sp. ANU 289, and *Rhizobium* sp. NGR234 (Table 1). The latter strain is known to have an unusually broad host range (Pueppke and Broughton 1999), whereas the remaining species are found in symbiotic interaction with alfalfa, common bean, and the non-legume *Parasponia*. In general, each plant tissue and genotype contained at least one *nifH* gene that might be of rhizobial origin, in addition to one *nifH*-containing bacterium not belonging to the  $\alpha$ -*Proteobacteria*. 'Muibai' contained only sequences of rhizobial origin (Table 1). Furthermore, highly similar or even identical sequences were found in different clones grown at distinct locations. Altogether these results suggest that the colonization of sweet potato by rhizobia is a common phenomenon, and it may even be that only a restricted range of species is able to proliferate inside the plant. Similarly, legumes are

usually nodulated by only one or several rhizobial species. Although rhizobia are famous for their symbiotic association with legumes, they also colonize a broad range of non-legumes endophytically (Reiter et al. 2002; Gutiérrez-Zamora and Martínez-Romero 2001; Yanni et al. 1997). To the best of our knowledge endophytic  $N_2$ -fixing activity has not been shown so far for rhizobia, but other plant growth promoting effects, such as phosphate solubilization and hormone production, have been reported (Sessitsch et al. 2002b; Yanni et al. 2001; Chabot et al. 1996; Höflich et al. 1995). For legume-cereal associations it has been suggested that rhizobia-enriched soil could serve as inoculum for non-legumes (Gutiérrez-Zamora and Martínez-Romero 2001; Yanni et al. 1997; Noel et al. 1996). In this study, plants were obtained from small fields where crop rotation with leguminous plants is common, possibly explaining the high abundance of rhizobial strains in sweet potato.

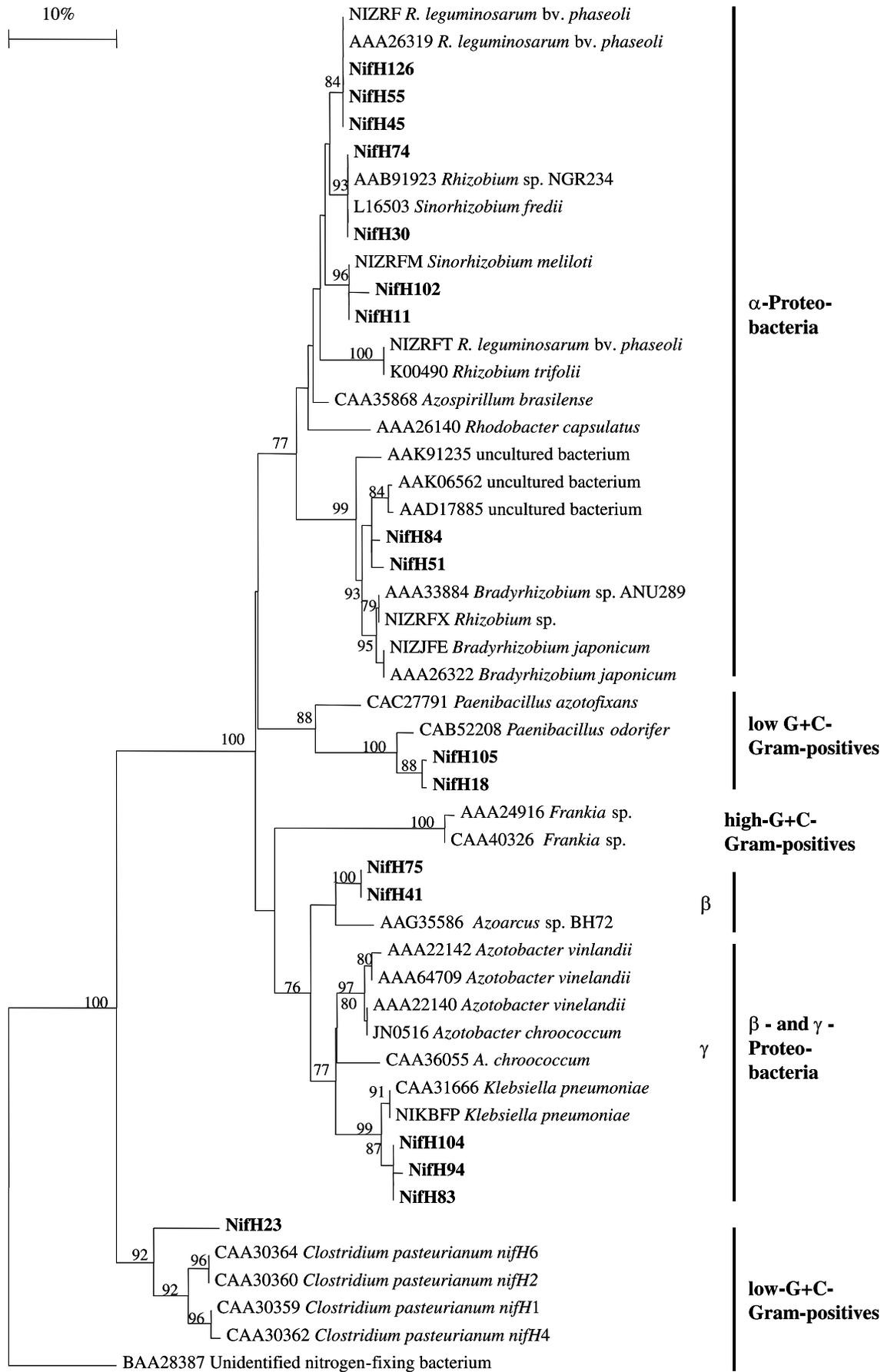
'Embu local' stems contained in high abundance (80%) a nitrogenase reductase gene with very high amino acid identity with *Azoarcus* sp. BH72 (Table 1). This strain colonizes Kallar grass and rice endophytically and is probably one of the few examples where *nifH* gene expression and  $N_2$  fixation in planta has been proven up-to-date (Hurek et al. 2002; Egener et al. 1999). Sequences with highest similarity to *Klebsiella pneumoniae* were found in some plants that were grown in the Nairobi region and in Embu (both Kenya). This *nifH* RFLP type represented 60%, 43%, and 83%, respectively, of the clones obtained from 'Guikanda' stems and tubers and from 'Embu local 2' stems (Table 1). *Klebsiella pneumoniae* is a common endophyte of maize (Chelius and Triplett 2001; McInroy and Kloepper 1995; Fisher et al. 1992), and at least some of these *Klebsiella* sp. endophytes are diazotrophic (Palus et al. 1996). Furthermore, production of a *K. pneumoniae* dinitrogenase reductase protein within maize roots has been demonstrated (Chelius and Triplett 2000). *Klebsiella* sp. have been also reported to colonize red clover (Sturz et al. 1998), grapevine (Bell et al. 1994), and rice (Elbeltagy et al. 2000).

Three NifH sequences were most likely related to bacteria within the low G+C Gram positives. Two of them were highly similar, although they were obtained from different sweet potato varieties grown in different countries and under different conditions, and clustered with  $N_2$ -fixing *Paenibacillus* species, including *P. azotofixans*, *P. polymyxa*,

**Fig. 2.** Neighbour-joining phylogenetic tree of partial NifH amino acid sequences, including the sequences obtained in this study. The database accession numbers are followed by the bacterial names. Bootstrap values greater than 70% are shown. The tree is based on the sequence information of 114 amino acids, whereas for BLAST analysis (Table 1) 123 amino acids were used, hence the minor differences between Table 1 and Fig. 2. The NifH sequences obtained in this study are in bold, and the database accession numbers are as follows: NifH11 AY159587, NifH18 AY159588, NifH23 AY159589, NifH30 AY159590, NifH41 AY159591, NifH45 AY159592, NifH51 AY159593, NifH55 AY159594, NifH74 AY159595, NifH75 AY159596, NifH83 AY159597, NifH84 AY159598, NifH94 AY159599, NifH102 AY159600, NifH104 AY159601, NifH105 AY159602, NifH126 AY159603.

**Table 1.** Similarities of sweet potato NifH amino acid sequences (123) to the most similar sequences from known diazotrophic bacteria.

Region/cultivar	Sequence	% abundance	Closest NCBI database match	% similarity	Phylogenetic position
Soroti, Uganda					
‘New Araka’					
Stem	NifHHyp8	100	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	88	$\alpha$ -Proteobacteria
Tuber	NifH11	71	<i>Sinorhizobium meliloti</i> NIZRFM	100	$\alpha$ -Proteobacteria
	NifH18	29	<i>Paenibacillus odorifer</i> CAB52208	96	Low G+C Gram-positives
‘Araka white’					
Stem	NifH23	83	<i>Clostridium pasteurianum</i> P09552	97	Low G+C Gram-positives
Tuber	NifH30	17	<i>Rhizobium</i> sp. NGR234 JS0238	78	$\alpha$ -Proteobacteria
	NifHHyp31	100	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	88	$\alpha$ -Proteobacteria
Embu, Kenya					
‘Embu local’					
Stem	NifH41	80	<i>Azoarcus</i> sp. BH72 AAG35586	98	$\beta$ -Proteobacteria
	NifH45	20	<i>Rhizobium etli</i> P00462	86	$\alpha$ -Proteobacteria
Tuber	NifH51	28	<i>Bradyrhizobium japonicum</i> P06117	99	$\alpha$ -Proteobacteria
	NifH55	44	<i>Rhizobium etli</i> P00462	86	$\alpha$ -Proteobacteria
	NifHHyp59	28	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	87	$\alpha$ -Proteobacteria
‘Embu local 1’					
Stem	NifHHyp67	100	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	87	$\alpha$ -Proteobacteria
Tuber	NifH74	29	<i>Rhizobium</i> sp. NGR234 NP_444112	78	$\alpha$ -Proteobacteria
	NifH75	71	<i>Azoarcus</i> sp. BH72 AAG35586	98	$\beta$ -Proteobacteria
‘Embu local 2’					
Stem	NifH83	83	<i>Klebsiella pneumoniae</i> P00458	85	$\gamma$ -Proteobacteria
	NifH84	17	<i>Bradyrhizobium</i> sp. ANU 289 P00463	98	$\alpha$ -Proteobacteria
Nairobi, Kenya					
‘Guikanda’					
Stem	NifHHyp93	40	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	85	$\alpha$ -Proteobacteria
Tuber	NifH94	60	<i>Klebsiella pneumoniae</i> P00458	85	$\gamma$ -Proteobacteria
	NifH102	14	<i>Sinorhizobium meliloti</i> NIZRFM	98	$\alpha$ -Proteobacteria
	NifHHyp103	14	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	87	$\alpha$ -Proteobacteria
	NifH104	43	<i>Klebsiella pneumoniae</i> P00458	85	$\gamma$ -Proteobacteria
	NifH105	29	<i>Paenibacillus odorifer</i> CAB52208	96	Low G+C Gram-positives
‘Muibai’					
Stem	NifHHyp112	100	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	87	$\alpha$ -Proteobacteria
Tuber	NifHHyp125	20	<i>Mesorhizobium loti</i> (hypothetical protein) NP_106264	87	$\alpha$ -Proteobacteria
	NifH126	80	<i>Rhizobium etli</i> P00462	86	$\alpha$ -Proteobacteria



*P. macerans*, and *P. odorifer* (Fig. 2). Nitrogen-fixing paenibacilli have been isolated mainly from the rhizosphere of grasses and maize (Seldin et al. 1984, 1998). One *nifH* sequence showed highest similarity to *Clostridium pasteurianum* (Table 1), an anaerobic N<sub>2</sub>-fixing bacterium that is not usually found in association with plants, at least by cultivation.

Several highly similar or even identical nitrogenase reductase sequences clustering with different bacterial genera and species, including *Sinorhizobium meliloti*, *Rhizobium* sp. NGR234, *Rhizobium etli*, *Klebsiella pneumoniae*, and *Paenibacillus odorifer*, could be detected in plants grown in distinct geographic locations. Furthermore, the climatic conditions were highly different. This indicates that these species are present in different African soils and are able to colonize sweet potato, irrespective of the plant genotype. The presence of *nifH*-containing bacterial sequences does not necessarily indicate that these genes were actually expressed. Nitrogen fixation by associative diazotrophs has been rarely proven, but these bacteria exert several other plant beneficial effects, such as synthesis of plant hormones and vitamins, improved nutrient uptake, enhanced stress resistance, and biocontrol of phytopathogens (Dobbelaere et al. 2003; Sessitsch et al. 2002b). However, it has been claimed that endophytic diazotrophs have an advantage over root-associated diazotrophs, as they are better placed to exploit the carbon substrates supplied by the plant. Furthermore, they colonize niches protected from oxygen, which is necessary for the expression and activity of nitrogenase (Dobbelaere et al. 2003). This may explain the N<sub>2</sub> fixation observed in sugarcane (Urquiaga et al. 1992), Kallar grass (Hurek et al. 2002), and sweet potato (Yoneyama et al. 1998). The results obtained from cultivation-independent analysis will direct and facilitate future attempts to isolate diazotrophic bacteria from sweet potato. In future studies we will investigate whether nitrogenases of diazotrophic bacteria are active in plants and whether they actually contribute to N<sub>2</sub> fixation.

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