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Rhizobium etli and *Rhizobium gallicum* Nodulate *Phaseolus vulgaris* in Egyptian Soils and Display Cultivar-Dependent Symbiotic Efficiency

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Abstract

Twelve rhizobial isolates were recovered from nodules of common bean (*Phaseolus vulgaris*) grown in two different locations of Egyptian soils. The most effective strains for nodule formation and nitrogen fixation were selected. Strain specificity with the bean cultivars Saxa, Canoca and Giza 6 from Germany, Colombia and Egypt were studied. The strains were characterized by amplified rDNA restriction analysis of 16S and 23S rDNA (ARDRA), plasmid DNA content and 16S rDNA sequencing. A high degree of genetic diversity was observed among the strains used. The strains were separated into three genotype groups. Genotype A was displayed by seven isolates classified as *Rhizobium etli*, while genotype B was displayed by a single isolate, classified as *R. gallicum*. Genotype C included four isolates which were unable to re-nodulate *Phaseolus vulgaris*, which were

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related to *Agrobacterium tumefaciens*. Single strains were further characterized by specific physiological tests and measurements such as acetylene reduction activity, nodule/root biomass ratio, shoot and root fresh weight.

Keywords: *Phaseolus vulgaris*, Egypt, rhizobia, molecular characterisation

1. Introduction

Phaseolus vulgaris has its origin in Central and South America. It is a major world crop with almost 20 million tons of seeds produced per year, ranking directly after soybeans and peanuts in the world production of grain legumes (FAOSTAT, 2001). The area on which common beans are produced is more than 27 million hectares, with an average production is around 7 dry ton (dt) per hectar (ha). High production rates are found in Germany with 26 dt per ha whereas the production rate in African countries is around 7 dt per ha. This means, there is a large potential for increasing the production of *Phaseolus vulgaris* in Africa (Kratzsch, 1999).

In Egypt, common bean is widely consumed as vegetables and also as dry seeds. The annually cultivated area ranges between 9–18 thousand hectares, yielding 15,000 tons of dry seeds and 150,000 tons of green pods (data from the Central Administration of Agriculture in Egypt CAAE, 1994). Different *Rhizobium* species are able to nodulate and fix nitrogen with the common bean (*Phaseolus vulgaris*), including *Rhizobium leguminosarum* bv. *phaseoli* (Jordan, 1984), *R. tropici* (Martinez et al., 1991), *R. etli* (Segovia et al., 1993), *R. gallicum* and *R. giardini* (Amarger et al., 1997). The percentage of biological nitrogen fixation on the N assimilation in *Phaseolus vulgaris* is, compared to other legumes, rather low, with 40–50% compared to 75% with faba beans, 70% with peas and up to 95% with lupines (Werner, 1999).

Failure of nodulation or lack of response to inoculation in the field has been reported, raising doubts about the benefits of inoculation (Buttery et al., 1987, Graham, 1981). Factors that can limit inoculation benefits are the promiscuity observed in *Phaseolus vulgaris* (Hernandez et al., 1995, Michiels et al., 1998), environmental conditions (Robert and Schmidt, 1983), the effect of high N-fertilizer concentrations used in intensive agriculture (Temprano et al., 1997) and genomic rearrangements in the micro-symbionts (Girard et al., 1991). The genetic biodiversity of *Rhizobium* in bean nodules is significantly affected by soil fertilization, especially by ammonia and nitrate (Caballero-Mellado and Martinez-Romero, 1999).

There are reports from different regions in the world stressing both genetic uniformity (Wegener et al., 2001) and large biodiversity of bean nodule isolates (Castro-Sowinski et al., 2002) but little information is available for Egyptian

bean-nodulating rhizobia. Based on these reports our research had the aim of (i) isolating new effective bean nodule isolates from Egyptian soils, characterizing their nodulation efficiency on bean cultivars from Egypt and other countries, (ii) to study the genetic diversity of these strains by molecular methods such as ARDRA techniques (Vinuesa et al., 1998), plasmid profiling (Kuykendall and Hashem, 1994) and to classify them on the basis of 16S rDNA phylogeny (Amarger et al., 1997).

2. Materials and Methods

Isolates and reference strains

Twelve rhizobial isolates were obtained from the root nodules of common bean (*Phaseolus vulgaris*) grown in two different locations of Egyptian soils. Isolates EBRI 2, 3, 6, 25 and 29 were from the Isma'ilya desert while isolates EBRI 20, 21, 23, 24, 26, 27 and 32 were from the Ashmun area in the Nile Valley Delta. Plant samples were collected by digging the root system with a block of soil. The moist blocks were transported in polyethylene bags to the laboratory for isolation of rhizobia. Rhizobia were isolated from surface-sterilized nodules according to the method described by Somasegaran and Hoben (1985). *R. tropici* CIAT 899T, *R. etli* CFN 42T and *A. tumefaciens* DSM30150 were obtained from culture collection of the Laboratory of Cell Biology and Applied Botany in Marburg. Strains of *R. gallicum* R602spT and *R. giardini* H152T were obtained from N. Amarger. Isolates and standard strains were preserved in 50% glycerol at –70°C for further studies.

Phenotypic characteristics

Strains were examined for growth on LB medium and on a basal medium with D-glucuronate as a main carbon source or with 5 μg ml^{-1} spectinomycin as done by Amarger et al. (1997). Also, strains were tested for growth on minimal medium MM containing malate or sorbitol as a sole carbon source, as reported by Segovia et al. (1993).

Bean cultivars

Three cultivars of *Phaseolus vulgaris* were used in this study. The Saxa and Canoca cultivars were obtained from the germplasm collection of the Department of Biology, Philipps-University Marburg, Germany and the Giza 6 cultivar was obtained from the Institute of Vegetable Crops Research, Ministry of Agriculture, Egypt.

Plant nodulation assays

The nodulation assays were performed in sterilized growth pouches or in Leonard jars with vermiculite: perlite (1:1 v:v) as substrate and N-free solution (Werner et al., 1975). Seeds of common bean were surface sterilized according to Vinuesa et al. (1998) and distributed on the surface of 1% agar plates and incubated for 3–4 days at 28°C for germination. After germination seedlings were transferred to growth pouches for nodulation experiments or to Leonard jars for host specificity experiments under sterile conditions. Seeds of *Leucaena leucocephala* cv. Cunningham were scarified for 20 min in concentrated H_2SO_4, surface sterilized and cultivated as previously described for common bean seeds (Vinuesa et al., 1998). Seeds were inoculated by adding 1 ml of rhizobial cultures at the mid of the exponential growth phase with about 10^8 cells ml^{-1} for each Leonard jar. Seeds cultivated in growth pouches, were inoculated by soaking for three minutes in one ml of a 1:20 diluted inoculum suspension.

Plants were cultivated in a controlled growth chamber with 15 h of light at 25°C and 9 h for darkness at 18°C and relative humidity of 75%. Light intensity was 14 k Lux. Plants were harvested 21 or 30 days after inoculation. Acetylene reduction assays (ARA) were performed as described by Bender and Rolfe (1985).

Isolation of DNA

Genomic DNA of the strains was obtained from liquid culture at the mid exponential phase using cetyltrimethyl amoniumbromide (Ausubel et al., 1994). Purified DNA was dissolved in T.E. buffer (10 mM Tris-HCl and 1 mM EDTA pH. 8.0). The concentration of DNA was adjusted to 50 µg ml^{-1}.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Primers fD1 and rD1 were used to amplify nearly full-length 16S rDNA genes (Weisburg et al., 1991), while the primers P3 and P4 were used to amplify the region of 23S rDNA described by Terefework et al. (1998). The PCR reaction was performed using the standard reaction mixture (50 µl) containing 1x PCR buffer, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 200 µM for each nucleotide (Boehringer GmbH, Mannheim, Germany), 15 pmol of each primer, 1 U of taq polymerase, and 50 ng of purified DNA. The temperature program was as follows: initial denaturation at 95°C for 3 min 30 s; 35 cycles of denaturation at 94°C for 70 s, anneling temperature at 56°C for 40 s and extension at 72°C for 130 s, and final extension at 72°C for 370 s (Vinuesa et al., 1998).

PCR products from 16S rDNA amplification experiments were digested with the four restriction enzymes *Hha1, Dde1, Msp1* and *Sau3a1,* while the amplified fragments of 23S rDNA amplicons were restricted with *Msp1, Sau3a1* and *Hinf1* (USB Amersham International), as recommended by the manufacturer.

The restriction patterns were resolved by electrophoresis in a 2% agarose gel in TBE buffer (Tris-Borate EDTA) at 55 v for 3 h, as described by (Vinuesa et al., 1998). The RFLP patterns were normalized using a 100 bp ladder from GIBCO BRL (Eggenstein, Germany). The gels were stained post-electrophoresis with ethidium bromide.

Analysis of rDNA restriction patterns

Gel images were digitised using a charge coupled device video camera (INTAS, Göttingen, Germany) and stored as TIFF files. Data were analysed using the Gel Compar II software package (version 2.0; Applied Maths, Kortrijk, Belgium) as described in detail elsewhere (Vinuesa et al., 1998).

Plasmid DNA content

Plasmid profile analysis for twelve rhizobial isolates EBRI 2, 3, 6, 20, 21, 23, 24, 25, 26, 27, 29 and 32 in addition to *R. tropici* (CIAT 899T), *R. gallicum* (R602spT) and *R. giardini* (H152T) and *R. etli* (CFN42T) was done according to the modified Eckhardt procedure of Kuykendall and Hashem (1994).

Direct sequencing of 16S rDNA

Amplified 16S rDNA fragments were purified using the Quiaquick high pure PCR purification kit (QIAGEN, Düsseldorf, Germany) according to manufacture's instructions. Primers fD1, rD1 and four internal primers (5' - CCG GGC GGT GTG TAC AGG G - 3'; 5' - TAC GGG AGG CAG CAG - 3'; 5' - GTC AAT TCC TTT GAG - 3' and 5' - CCG GGC GGT GTG TAC AAG G - 3') were used to obtain full-length readings of both strands of the *rrs* amplicon. Cycle-sequencing was performed using the AmpliTaq-DNA-polymerase, based on the dideoxy chain terminator technique of Sanger et al. (1977). DNA sequences were read on a 373A (Applied Biosystems). Sequence reads were edited and assembled using DNASTAR (Lasergene) software. The 16S rDNA sequence for 8 Egyptian isolates were deposited at GeneBank under the accession numbers from AY 221174 to AY 221181, corresponding to isolates EBRI 2, EBRI 3, EBRI 21, EBRI 26, EBRI 32, EBRI 27, EBRI 20 and EBRI 25, respectively.

16S rDNA sequence alignment and phylogeny estimation

Sequence similarity searches were performed at the NCBI server using BLASTN (http://www.ncbi.nlm.nih.gov/blast). Multiple nucleotide sequence alignments were generated and edited using ClustalW, as implemented in BioEdit (Hall, 1999). The *rrs* multiple sequence alignment was manually adjusted to fit that produced by the Ribosomal Database Project-II (Maidak et al., 2001). Model fitting was performed by likelihood ratio tests (LRTs) as implemented in DAMBE (Xia and Xie, 2001). A neighbour joining (NJ) phylogeny was inferred with the model selected by LRTs using MEGA2.1 (Kumar et al., 2001) and the complete gap deletion option. The robustness of the phylogeny was assessed by non-parametric bootstrapping with 1000 pseudoreplicates.

nodC amplification

Primers nodCF2 and nodCI, and amplification protocol of Laguerre et al. (2001) were used for *nodC* amplification experiments.

Statistical analysis

Data of nodulation assays were analysed using the least significant difference test according to Sendecor and Cochran (1978).

3. Results

Efficiency and host specificity of rhizobial strains

Twelve rhizobial isolates were recovered from the root nodules of *Phaseolus vulgaris* plants sampled from two regions in Egypt. Seven isolates were from the Ashmun area in the middle of the Delta-Nile Valley and five were from the Isma'ilya desert. Nodulation phenotypes for these isolates with cultivar Saxa are summarized in Table 1.

The best strain for nodule formation was EBRI 3 (129 nodules plant^{-1}) followed by EBRI 29 and CIAT 899T. Nodule fresh weight was also at its highest with strain EBRI 3, followed by EBRI 29 and EBRI 21. Plants inoculated by strain EBRI 21, 24, 3 and CIAT 899T produced the highest shoot biomass compared to control plants. Noteworthy, the weight of nodules formed by strains EBRI 3, 27 and 29 were higher than the weight of the root system after detaching the nodules (Table 1). Isolate EBRI 21 gave the highest ARA

activity with 13.7 nM C_2H_4 h^{-1} mg^{-1} nodule followed by strains EBRI 2 and EBRI 26.

Based on these results we selected the strains EBRI 2, 21 and 26 as compared to the reference strain *R. tropici* CIAT 899T to study the N_2 fixation capacity of these strains with two other cultivars, Canoca and Giza 6, at 30 days after planting (Table 2). With the cultivar Giza 6, the strains from Egypt formed the largest number of nodules and the highest nodule biomass. Strains CIAT 899T and EBRI 26 were most effective strains for nitrogen fixation. They produced 16.9 and 13.2 nM C_2H_4 h^{-1} mg^{-1} nodule. With cultivar Canoca from Colombia, the South American strain CIAT 899T of *Rhizobium tropici* gave the highest nodule biomass compared to the three strains from Egypt. With both cultivars tested in this series, only strain EBRI 2 produced consistently a nodule/root biomass ratio of under 1, whereas for the other three strains this ratio was above 1.

Table 1. Nodulation data of new isolates of bean rhizobia nodulating *Phaseolus vulgaris* cv. Saxa, 21 days after planting.

Treatment	No. of nodules plant^{-1}	FW of nodules mg plant^{-1}	FW of shoot g plant^{-1}	FW of root mg plant^{-1}	nMol C_2H_4 h^{-1} mg^{-1} nodules	Ratio of nodule to root FW
Control	0	0	1.8	1,250	0	–
CIAT 899	71	578	2.4	830	6.5	0.70
EBRI 2	67	510	2.1	1,140	8.9	0.45
EBRI 3	129	907	2.4	870	3.7	1.04
EBRI 6	0	0	1.9	780	0	–
EBRI 20	0	0	1.8	260	0	–
EBRI 21	73	695	2.6	1,160	13.7	0.56
EBRI 23	0	0	1.7	340	0.0	–
EBRI 24	60	670	2.5	690	7.5	0.97
EBRI 25	0	0	1.9	900	0	–
EBRI 26	62	507	2.4	930	7.9	0.55
EBRI 27	67	561	2.2	350	6.2	1.60
EBRI 29	79	715	2.2	290	6.4	2.46
EBRI 32	52	600	2.0	650	Nd	0.92
L.S.D 0.05	27.8	281	Ns	240	3.82	–

Results are taken from three replicates for each parameter. EBRI: Egyptian bean rhizobial isolates. Ns: non significant result. Nd: not determined.

Table 2. Nodulation data of selected isolates of bean rhizobia with different host cultivars of *P. vulgaris*, 30 days after planting.

Treatment	No. of nodules plant^{-1}	FW of nodules mg plant^{-1}	FW of shoot g plant^{-1}	FW of root mg plant^{-1}	nMol C_2H_4 h^{-1} mg^{-1} nodules	Ratio of nodule to root FW
Giza 6						
Control	0	0	5.1	2,050	0	–
CIAT 899	132	812	9.8	390	16.7	2.1
EBRI 2	192	1,692	14.4	2,300	7.1	0.73
EBRI 21	170	991	12.3	890	9.5	1.11
EBRI 26	165	896	10.8	800	13.2	1.12
Canoca						
Control	0	0	2.3	1,390	0	–
CIAT 899	153	1,289	10.1	800	9.4	1.61
EBRI 2	91	838	8.3	1,310	14.3	0.63
EBRI 21	161	772	9.0	490	7.6	1.58
EBRI 26	203	948	8.3	790	12.7	1.20
L.S.D 0.05	62.91	460	3.57	530	5.02	–

Molecular characterization

Amplified ribosomal DNA restriction analysis (ARDRA) of 16S and 23S rDNA fragments, and phenotypic characteristics

All Egyptian isolates and standard strains yielded *rrs* amplification products of about 1,500 bp and *rrl* amplicons of about 2,100 bp. The *rrs* amplicons were subjected to digestion with enzymes *Hha1*, *Dde1*, *Msp1* and *Sau3a1* while the products from the locus *rrl* were restricted with *Hinf1*, *Msp1* and *Sau3a1*. A Dice/UPGMA analysis of the combined restriction patterns from the 16S and 23S amplicons resulted in the dendrogram shown in Fig. 1.

Three major genotypes (A, B and C) were identified among the 12 Egyptian bean isolates. Strains with genotype A (EBRI 2, 3, 21, 24, 26, 27 and 29) formed the predominant group which was related to *R. etli* CFN42T. These strains were able to grow on MM medium with malate as a sole carbon source but were unable to grow on the same medium with sorbitol as a main carbon source (Table 3). Similar results were reported by Segovia et al. (1993). Strain EBRI 32, which nodulated *Phaseolus vulgaris* (Table 1) and *Leucaena leucocephala* (Table 4) had 16S and 23S rDNA ARDRA patterns identical to those from *R. gallicum* R602spT, forming group B.

Table 3. Phenotypic characteristics of rhizobial strains on different media.

Strain	Growth on LB medium	Growth on MM medium with		Growth on basal medium with	
		Malate	Sorbitol	D-Glucuronate	5 μg ml^{-1} spectinomycin
R. etli CFN42T	–	+	–	+	–
EBRI 2	–	+	–	+	+
EBRI 3	–	+	–	+	–
EBRI 6	+	+	+	+	+
EBRI 20	+	+	+	+	+
EBRI 21	–	+	–	–	–
EBRI 23	+	+	+	+	+
EBRI 24	–	+	–	+	+
EBRI 25	+	+	+	+	+
EBRI 26	–	+	–	+	+
EBRI 27	–	+	–	–	–
EBRI 29	–	+	–	–	+
EBRI 32	–	+	+	–	–
R. gallicum R602spT	–	+	+	–	–
R. tropici CIAT899T	+	+	+	+	+
R. giardinii H152T	–	+	+	+	+

+: positive growth. –: no growth.

Strain EBRI 32 was not able to grow on basal medium containing glucuronic acid as a sole carbon source or with 5 μg ml^{-1} spectinomycin, and was not able to grow on LB medium (Table 3) as previously mentioned for *R. gallicum* (Amarger et al., 1997). Finally, isolates EBRI 6, 20, 23 and 25 presented RFLP patterns highly related to those from *A. tumefaciens* DSM 30150, forming group C. These four strains failed to re-nodulate *Phaseolus vulgaris* cultivar Saxa (Table 1) and were able to survive on LB medium as reported by Amarger et al. (1997).

Plasmid DNA content

Fourteen plasmid patterns were identified among the 16 rhizobial strains tested (Table 4). Plasmid numbers varied from 2 to 6 and their sizes ranged from 40 kb to 650 kb. All strains grouped in the ARDRA cluster A contained a large plasmid of about 650 kb, whereas the largest plasmid found in strains from

Table 4. Nodulation of legume tree and plasmid DNA content of rhizobial strains used in this study.

Strain	Nodulated *L. leucocephala*	Size of plasmid kb	ARDRA cluster
R. etli CFN42T	–	650–510–390–270–180–150	
EBRI 2	–	650–510–400–390–260–65	A
EBRI 3	–	650–400–390–180	A
EBRI 6	–	250–140–108	C
EBRI 20	–	150–65–45	C
EBRI 21	–	650–490–250	A
EBRI 23	–	270–115–65–45	C
EBRI 24	–	650–510–390–250–190	A
EBRI 27			
EBRI 25	–	380–250–40	C
EBRI 26	–	650–510–390–250–208	A
EBRI 29	–	>1.500–650–400–390–180	A
EBRI 32	+	550–250	B
R. gallicum R602spT			
R. tropici CIAT899T	+	>1.500–550–250	
R. giardinii H152T	+	140–120	

+: able to nodulate *L. leucocephala*. –: unable to nodulate *L. leucocephala*.

cluster B was 550 kb, and that found in strains from cluster C only 380 kb. Plasmid profile analysis provided higher taxonomic resolution than the RFLP analysis. Each strain had a unique plasmid profile except for the pair EBRI 24 and EBRI 27, which shared the same plasmid patterns. Irrespective of the megaplasmids, strain EBRI 32 displayed the same plasmid profile as strains *R. tropici* CIAT 899T and *R. gallicum* R602spT. It should be noted that the megaplasmids present in the two later strains (Silva et al., 2003) could be detected only for *R. tropici* CIAT 899T and *R. etli* EBRI 29 but could not be resolved for *R. gallicum* R602spT in our gels.

Phylogenetic analysis of rrs sequences

Fig. 2 shows the phylogenetic placement of eight Egyptian bean nodule isolates in the context of a neighbour-joining (NJ) phylogeny inferred from the *rrs* sequences of all rhizobial type strains and those from closely related non-rhizobial species. Due to the bias in base composition and transitional substitutions present in the data set, the Tamura-Nei (TrN93), a model of nucleotide substitution was used to reconstruct the phylogeny, which provided a highly significant ($P<0.000001$; DF=2) improvement in the fit of the model to

the data ($-LnL_{TrN93}$=7509.9307) over the Jukes-Cantor (JC) model (LnL_{JC}=7615.3545).

Model fitting was further improved with very high statistical significance (LRT= 212.0586; P<0.00001) by accounting for among-site rate variation using a discrete gamma distribution with 8 rate categories, with an estimated shape parameter of value α=0.15 (-LnLTrN+G=7403.9014). Therefore, the NJ phylogeny shown in Fig. 2 was reconstructed using the TrN+G model. This phylogeny confirms the ARDRA results. Strains EBRI 2, 3, 21, 26 and 27 form a highly supported clade along with *R. etli* CFN 42T. Strains EBRI 20 and 25 are significantly associated to the lineages of *R. radiobacter* and *R. rubi* (formally *A. radiobacter* and *A. rubi*), respectively.

PCR amplification of nodC gene fragments

All strains yielded *nodC* amplification products of about 1,450 bp except the *Agrobacterium*-like isolates (data not shown). Seven strains yielded two fragments, while strains EBRI 21, 27, R602sp and H152 produced only one fragment.

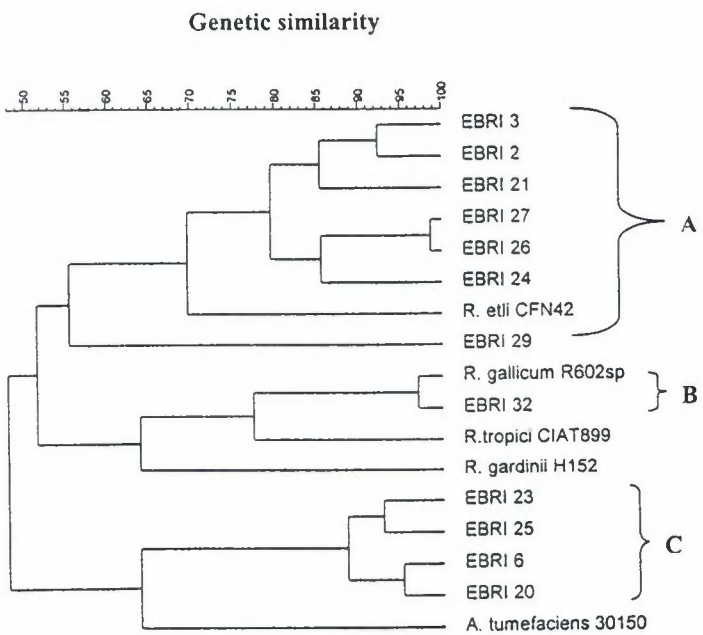

Figure 1. DICE/UPGMA analysis of 16S and 23S rDNA amplicons from twelve Egyptian isolates and 5 reference strains. The 16S rDNA amplification products were digested with enzymes *Hha1*, *Dde1*, *Msp1* and *Sau3a1*, and the 23S rDNA amplicons with *Hinf1*, *Msp1* and *Sau3a1*.

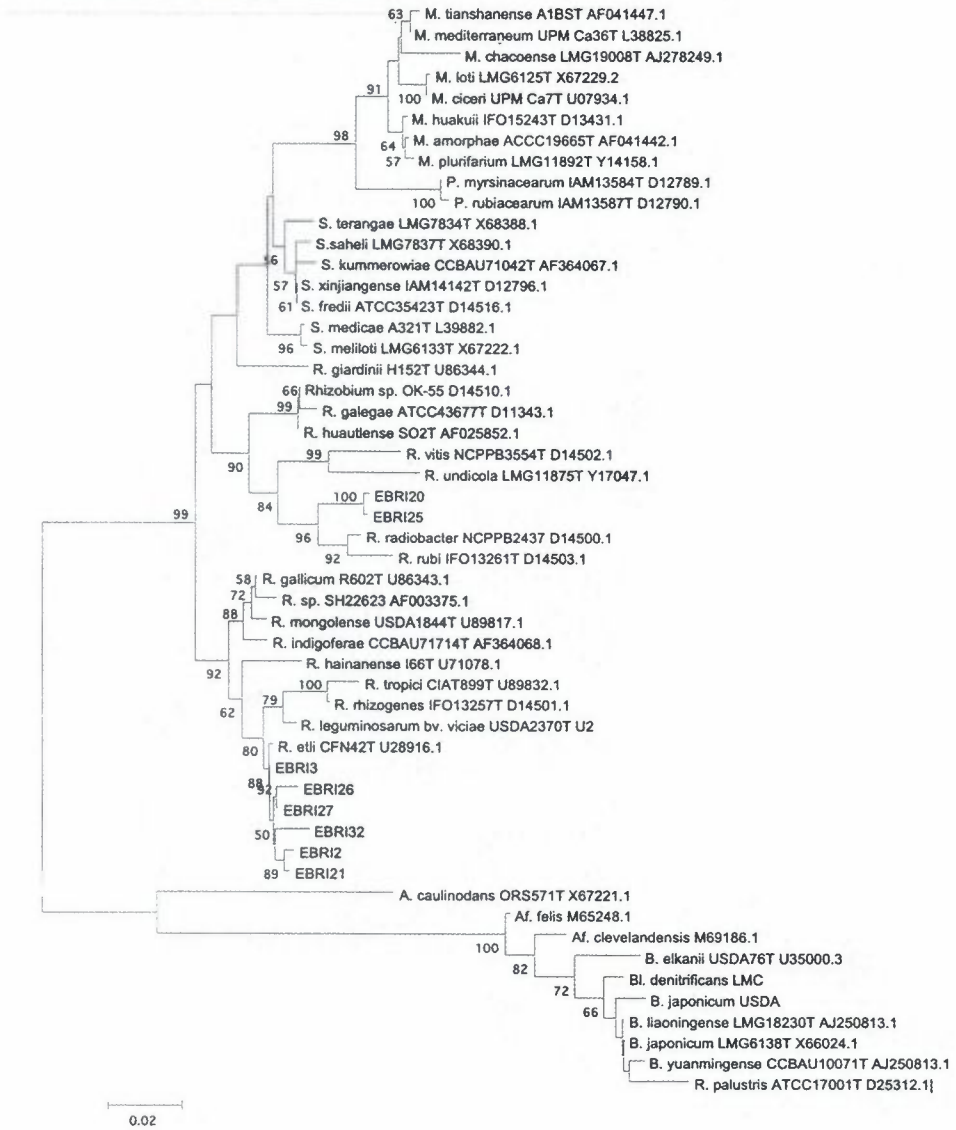

Figure 2. Neighbour-joining phylogram (TrN93+G, α=0.15) based on 1,300 bp of aligned *rrs* sequences from 6 Egyptian and 44 reference strains. Bootstrap support values greater than 50% (out of 1000 pseudoreplicates) are indicated at the branching points. Numbers on the left side are the accession numbers for the sequences used.

Similar results were reported by Mhamdi et al. (2002). ARDRA, 16S rDNA phylogeny, nodulation assays and plasmid profiling experiments consistently indicated that isolates EBRI 6, 20, 23 and 25 were closely related to the *A. radiobacter* lineages. These were the only strains that did not yield *nodC* amplification products, suggesting that these are not symbiotic isolates, which is consistent with their inability to nodulate *P. vulgaris* (Table 1).

4. Discussion

Phaseolus vulgaris nodules can fix about 40–50% of the plant's N-demand by symbiotic N_2 fixation (Werner, 1992), but efficiencies in the field are often much lower. This is partly due to the relatively broad host-range symbiotic association exhibited by common bean, which is known to nodulate in nature with at least five *Rhizobium* species of contrasting symbiotic competitiveness and efficiency (Martinez-Romero, 2003; Mhamdi et al., 1999; Silva et al., 1999; Silva et al., 2003). It is therefore advantageous to select effective strains for specific cultivars from the region where the beans are grown. To achieve this goal, 12 strains were isolated from two geographic regions of Egypt to characterize them taxonomically as well as to evaluate some of their key symbiotic phenotypes.

The genetic diversity of the EBRI isolates was first analysed by combined 16S and 23S ARDRA, which revealed that most of the isolates (58%) were closely related to *R. etli*. Four isolates were related to *A. tumefaciens* (*R. radiobacter*), whereas only one isolate was related to *R. gallicum*. This was confirmed by phylogenetic analysis of nearly full-length 16S rDNA sequences, the key gene used in current rhizobial systematics for species identification (Sawada et al., 2003). Strain EBRI 32, which was grouped with *R. gallicum* in the ARDRA, had a nodulation phenotype and a plasmid profile similar to those exhibited in *R. gallicum* R602sp but clustered with the genetic lineage of *R. etli* (Fig. 2) suggesting a possible gene transfer, causing heterogeneity in *rrs* phylogeny (Ueda et al., 1999). Plasmid profiles also differentiated these three major lineages of isolates, as did discriminatory phenotypic characters such as growth on LB medium, growth on basal medium with glucuronic acid as a main carbon source or addition of 5 μg ml^{-1} spectinomycin, growth on MM medium with malate or sorbitol as a carbon source, colony morphology and nodulation parameters.

Based on these data we can conclude, that the isolates analysed in this study correspond to *R. etli*, *R. gallicum* and a *Rhizobium* lineage closely related to *R. radiobacter* (former *A. tumefaciens*). These results are consistent with those reported for bean nodule isolates from Tunisia, (Mhamdi et al., 1999; 2002) and from Central and West Africa (Diouf et al., 2000) who found that *R. etli* is the

predominant common bean micro-symbiont in these soils. Although the latter authors did not find *Agrobacterium*-like isolates, others have isolated such bacteria from diverse hosts, including common bean nodules from Morocco (Drevon et al., 2001), from Tunisia (Mhamdi et al., 2002), from legume nodules from Pakistan (Hameed et al., 2004) and nodules from tropical legumes (de Lajudie et al., 1999). The *Agrobacterium*-like isolates seem to be symbiotically unstable under laboratory conditions, loosing the ability to nodulate the host, which is likely due to loss of key symbiotic genes or the entire pSym as evidenced by our *nodC* PCR ampilification and plasmid profiling experiments.

The symbiotic instability of *Agrobacterium*-like isolates has been observed by other workers (de Lajudie et al., 1999; Martinez et al., 1987) and this may be the most probable explanation for the loss of symbiotic phenotype of isolates EBRI 6, 20, 23 and 25. Such isolates are therefore a poor choice for the formulation of common bean inoculants. Hungria and Araujo (1995) reported that the strain SEMIA 4064, used as a commercial inoculant lost its ability to fix nitrogen with common bean under field and green house conditions. These observations indicate that inoculation programs should be directed not only to select effective rhizobial strains but also to select strains have genetic stability to avoid to lose the pSym plasmid or genomic rearrangements (Flores et al., 2000).

The initial nodulation experiments using the German *P. vulgaris* cv. Saxa germplasm, the Colombian cultivar Canoca and the Egyptian cultivar Giza 6 revealed that there is remarkable degree of host-preference for each strain. Nodulation experiments with *Leucaena leucocephala* cv. Cunningham, indicate that only strain (EBRI 32) was able to nodulate this tree legume and failed to grow on LB medium, which is consistent with its classification as *R. gallicum* (Amarger et al., 1997).

In conclusion, effective *R. etli* and *R. gallicum* isolates were obtained from field grown common bean nodules in Egypt, which exhibited contrasting host-dependent symbiotic efficiencies. Our next studies will focus on nodulation competitiveness with the ultimate goal of developing a multi-strain inoculum for *P. vulgaris* in Egyptian soil.

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