RESEARCH ARTICLE

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

International Microbiology (2013) 16:157-163
doi: 10.2436/20.1501.01.190 ISSN 1139-6709 **www.im.microbios.org**

Identification of rhizobial strains nodulating Egyptian grain legumes

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Received 27 July 2013 · Accepted 25 September 2013

Summary. Fifty four bacterial strains were isolated from root nodules of the grain legumes *Cicer arietinum*, *Lens esculentus, Phaseolus vulgaris*, *Pisum sativum*, and *Vicia faba* grown in cultivated lands of Beni-Suef Governorate (Egypt). Repetitive extragenic palindromic (REP)-polymerase chain reaction (PCR) clustered the strains into 15 REP-PCR groups. The nearly complete sequence of the 16S rRNA gene from a representative strain of each REP-PCR pattern showed that the strains were closely related to members of the family Rhizobiaceae of the Alphaproteobacteria. Pairwise alignments between globally aligned sequences indicated that the strains from *V. faba* had 99.6 % identity with *Rhizobium leguminosarum*, and those from *P. vulgaris* 99.76 % and 100 % with sequences from *R. leguminosarum* and *R. mesosinicum*, respectively. Strains from *P. sativum* had 99.76 %, 99.84 %, and 99.92 % sequence identity with *R. leguminosarum*, *R. etli*, and *R. pisi*, respectively, and those from *L. esculentus* had 99.61 % identity with sequences from *R. leguminosarum*. Sequences of the strains from *C. arietinum* had 100 % identity with those of *Mesorhizobium amorphae* and *M. robiniae*, respectively. Nitrogenase activity, determined as acetylene-dependent ethylene production, was detected in nodules formed after inoculation of the corresponding host plant with the representative rhizobial species. [**Int Microbiol** 2013; 16(3):157-163]

Keywords: *Rhizobium* · *Mesorhizobium* · legumes · 16S rRNA gene · phylogenetic trees

Introduction

Nitrogen is the most significant yield-limiting element in many agricultural production systems. External inputs of nitrogen to agriculture may come from mineral fertilizers, the production of which is heavily dependent on fossil fuels. Alternatively, nitrogen can be obtained from symbiotic nitrogen fixation by nodule-forming legume and non-legume associated rhizobial and actinorhizal bacteria, respectively [28].

Members of the Leguminosae (Fabaceae) comprise 17,000 to 19,000 species and play an important ecological role, with representatives in nearly every terrestrial biome on Earth [17]. These plants are best characterized by their ability to establish N_2-fixing symbiotic associations with Alphaproteobacteria of the genus *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Ensifer* [10,24,39], collectively referred to as rhizobia. Other non-rhizobial genera have been shown to nodulate legumes [4,23,29,35,37], which can also be nodulated by Betaproteobacteria, specifically, the genera

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Burkholderia and *Cupriavidus* [3,5,8,11,19,30]. During the infection process, an exchange of molecular signals occurs between the two partners, leading to the formation of root nodules, where nitrogen fixation takes place [32]. Because of this ability, legumes can grow in arid, nitrogen-deficient soils, acting as pioneer plants for soil stabilization and colonization, enhancing their fertility, and, consequently, preventing erosion and desertification. Despite potential errors in accurate calculations of N_2 fixation at global scales, an overall estimate of 50–70 Tg of biologically fixed N in agricultural systems has been estimated [13].

Since the use of rhizobia as a biofertilizer is a friendly environmental alternative to mineral fertilization, inoculation of legumes is a common agricultural practice, including in Egypt, especially in the country's newly reclaimed soils [45,46]. Nearly 95 % of Egypt's land surface can be categorized as arid and semi-arid. In these ecosystems, abiotic stresses, such as salinity or drought, limit legume cultivation and therefore crop production [45,46]. In Egypt, the grain legumes chickpea (*C. arietinum*), lentil (*L. esculentus*), common bean (*P. vulgaris*), pea (*P. sativum*), and broad bean (*V. faba*) are widely cultivated all along the Nile River for human consumption. In previous studies, *R. etli* and *R. gallicum* were isolated from root nodules of *P. vulgaris* plants growing in the Ismailia desert and the Ashmun area of the Nile Valley and Nile Delta [25]. Phylogenetic analyses based on partial sequencing of the 16S rRNA gene of 34 free-living rhizobial strains directly isolated from soils taken at the same locations identified 38.2 % of the strains as *E. meliloti*, 29.4 % as highly related to *E. medicae*, 23.5 % as *Agrobacterium tumefaciens*, and 8.8 % as taxonomically similar to *R. etli* [26]. Recently, the phenotypic characteristics and nodulation capacity of more than 50 rhizobial-like strains, isolated from the root nodules of lentils, common beans, peas, chickpeas, and broad beans, have been described [47]. Here we report on the identification of those strains on the basis of their 16S rRNA gene phylogenies. Our data contribute to further characterizing endosymbiotic bacteria associated with Egyptian grain legumes.

Materials and methods

Isolation of bacteria from nodules and culture conditions. Nodules (10/plant) were collected from roots of agriculturally grown, healthy *C. arietinum*, *L. esculentus*, *P. vulgaris, P. sativum*, and *V. faba* plants near the towns of Tezmant and Beni-Suef (Beni-Suef Governorate, Middle Egypt) (20 plants/location), where they are agriculturally-grown. Nodules were surface-sterilized as indicated earlier [47], placed independently in Petri dishes, and crushed in a drop of sterile water with a sterile glass rod. The resulting suspension was streaked onto Petri dishes containing yeast extract-mannitol (YEM) medium [42] supplemented with 0.025 g Congo Red/l. After incubation of the plates at 30 ºC for 7 days, colony-forming units, which represented all of the colony types that could be distinguished by microscopic observation, were chosen. All rhizobial strains used in this study were routinely grown on YEM medium.

DNA extraction and PCR amplifications. For DNA extraction and PCR amplifications, genomic DNA was isolated from bacterial cells using the RealPure Genomic DNA extraction kit (Durviz, Spain), according to the manufacturer's instructions. The quantity of DNA was determined using a Nanodrop spectrophotometer (NanoDrop ND1000, Thermo Fisher Scientific, USA). Repetitive extragenic palindromic (REP)-polymerase chain reactions (PCR) were performed using primers REPIR-I and REP2-I, according to de Bruijn [6]. PCR amplifications of 16S rRNA gene fragments were carried out using the two opposing primers 41f and 1488r as previously reported [12]. Amplification products were purified using the Qiagen PCR product purification system and subjected to cycle sequencing using the same primers as for PCR amplification, with ABI Prism dye chemistry. The products were analyzed with a 3130 × l automatic sequencer at the sequencing facilities of Estación Experimental del Zaidin, CSIC, Granada, Spain. The obtained sequences were compared to those in the GenBank database using the BLAST program [1] and with the sequences held in the EzTaxon-e server [15]. The sequences were aligned using Clustal W software [33]. The distances were calculated according to Kimura's two-parameter model [16]. Phylogenetic trees were inferred based on the maximum likelihood (ML) method [9], using MEGA 5.0 software [31].

Plant nodulation tests and nitrogenase activity. Seeds of *C. arietinum, L. esculentus, P. vulgaris, P. sativum*, and *V. faba* were surface-sterilized as above and allowed to germinate at 30 °C in the dark. Seedlings (1–4/pot) were planted in 1/2-kg pots containing sterile sand and vermiculite (1:1, v:v) and inoculated separately with each of the 15 strains. Uninoculated plants were used as a control for nodulation experiments. Plants were grown under natural daylight supplemented with artificial lighting, fed with N-free mineral solution [22], and harvested at 10 % flowering to check for nodule formation. Nitrogenase activity was determined as acetylene-dependent ethylene production, as described previously [47].

Accession numbers. Accession numbers of the nucleotide sequences of the rhizobial species used in this study are shown in the figure trees.

Results

REP-PCR and 16S rRNA gene phylogenetic analysis. Fifty-four bacterial strains, 12 from *P. sativum*, 11 each from *C. arietinum* and *V. faba*, and 10 each from *L. esculentum* and *P. vulgaris*, were isolated from extracts of nodules taken from healthy, agriculturally-grown plants in Beni-Suef Governorate (Egypt) [47].

The 54 isolates were represented by 15 different REP-PCR patterns (Table 1). The nearly complete sequence of the 16S rRNA gene from a representative strain of each REP pattern revealed that all of the isolates were members of the family Rhizobiaceae of the Alphaproteobacteria, of which 12 belonged to the genus *Rhizobium* and three to the

Table 1. Phylogenetic classification of bacterial strains isolated in this study

Strains[a]	REP-PCR pattern	Closest related genus[b]	Family
BSPV1, **BSPV2**, BSPV3, BSPV4, BSPV5	I	*Rhizobium*	Rhizobiaceae
BSPV6, **BSPV7**	II	*Rhizobium*	Rhizobiaceae
BSPV9	III	*Rhizobium*	Rhizobiaceae
BSPV11, BSPV12	IV	*Rhizobium*	Rhizobiaceae
BSPS1, BSPS2, BSPS3, **BSPS4**, BSPS5, BSPS6	V	*Rhizobium*	Rhizobiaceae
BSPS7, BSPS8, BSPS9	VI	*Rhizobium*	Rhizobiaceae
BSPS10, BSPS11, BSPS12	VII	*Rhizobium*	Rhizobiaceae
BSCA1, BSCA2	VIII	*Mesorhizobium*	Rhizobiaceae
BSCA3, BSCA4, **BSCA8**, BSCA11	IX	*Mesorhizobium*	Rhizobiaceae
BSCA5, BSCA6, BSCA7, **BSCA9**, BSCA10	X	*Mesorhizobium*	Rhizobiaceae
BSVF1, **BSVF2**	XI	*Rhizobium*	Rhizobiaceae
BSVF3, BSVF4, **BSVF5**, BSVF6, BSVF7	XII	*Rhizobium*	Rhizobiaceae
BSVF8, **BSVF9**, BSVF10, BSVF11	XIII	*Rhizobium*	Rhizobiaceae
BSLE1, BSLE3, **BSLE4**, BSLE5, BSLE6, BSLE7, BSLE8	XIV	*Rhizobium*	Rhizobiaceae
BSLE9, **BSLE10**, BSLE11	XV	*Rhizobium*	Rhizobiaceae

[a]Strains named BS to indicate Beni-Suef Governorate, followed by the letters PV, PS, CA, VF, and LE, representing *P. vulgaris*, *P. sativum*, *C. arietinum*, *V. faba*, and *L. esculentum*, respectively. Strains shown in bold were chosen as the representative strains of each REP-PCR group.
[b]Based on the 16S rRNA gene.

Mesorhizobium group (Table 1). The ML phylogenetic tree (Fig. 1) and EzTaxon-e analysis (Table 2) inferred from the 16S rRNA genes sequences indicated that strains BSPV2, BSPV7, BSPS4, BSVF2, BSVF5, BSVF9, BSLE4, and BSLE10 clustered with *R. leguminosarum* USDA 2370^T, based on identity values > 99.6 %, and that strains BSPS7, BSPS10, and BSPV9 grouped with *R. pisi* DSM 30132^T, *R. etli* CFN 42^T, and *R. mesosinicum* CCBAU 25010^T, respectively, with identity values > 99.8 % in all cases. Strain BSCA1 clustered with *M. amorphae* ACCC 19665^T, and strains BSCA8 and BSCA9 with *M. robiniae* CCNWYC 115^T. These three strains had 100 % identity with the 16S rRNA gene sequences of their corresponding type strain.

Plant nodulation tests. The 15 rhizobial strains identified in this study nodulated their original host plants. Nodules fixed N_2, with nitrogenase activity values, determined as acetylene-dependent ethylene production, varying from 51 nmol C_2H_2 plant^{-1} h^{-1} in *C. arietinum* nodulated by strain BSCA8 to 480 nmol C_2H_2 plant^{-1} h^{-1} in *P. vulgaris* inoculated with strain BSPV2.

Discussion

In this study, rhizobial bacteria from root nodules of the grain legumes *C. arietinum*, *L. esculentus, P. vulgaris*, *P. sativum*, and *V. faba* growing in cultivated lands of the Beni-Suef Governorate (Egypt) were identified. REP-PCR fingerprinting was used to group the strains. This technique has been extensively used to cluster bacteria at the subspecies or strain level [6,40] and is known to be a powerful tool for studies on microbial ecology and evolution [14].

Phaseolus vulgaris is a promiscuous legume able to form symbioses with several species of *Rhizobium*, including *R. leguminosarum*, *R. etli*, *R. gallicum, R. giardinii*, and

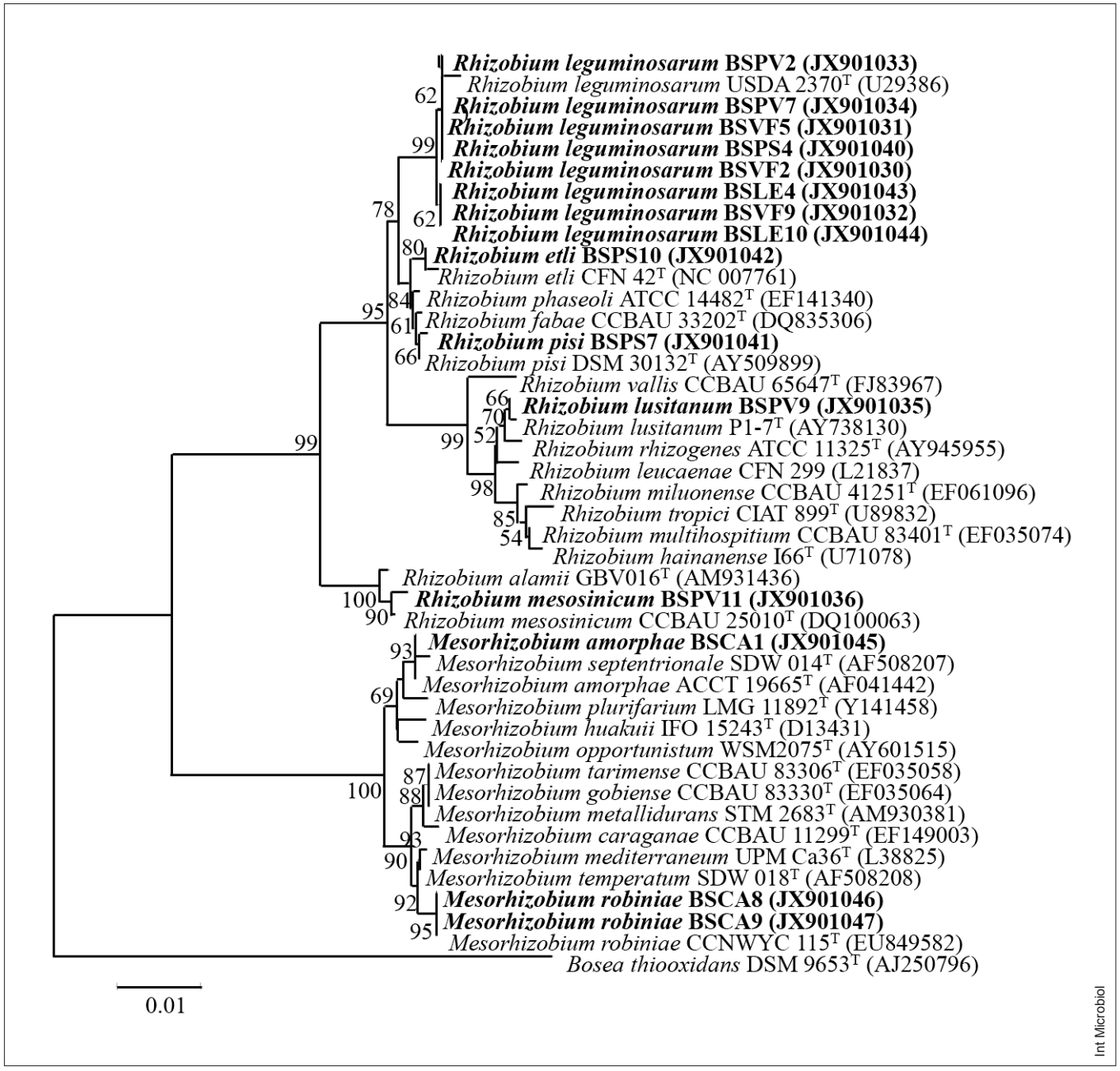

Fig. 1. Maximum likelihood phylogenetic tree based on partial 16S rRNA gene sequences of strains from nodules of *P. vulgaris*, *P. sativum*, *C. arietinum*, *V. faba*, and *L. esculentus* and phylogenetically related species within the genera *Rhizobium* and *Mesorhizobium*. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Values lower than 70 are not shown. Bar, 1 substitution per 100 nucleotide position. The tree is rooted on *Bosea thiooxidans* DSM 9653.

R. tropici [10,24]. We found that common beans were nodulated by *R. leguminosarum* as well as by *R. lusitanum* and *R. mesosinicum* (Table 2). Nodulation of *P. vulgaris* by *R. leguminosarum* [10, 24] and *R. lusitanum* [38] is well established. Our results extend those data with the finding that *P. vulgaris* can be nodulated by *R. mesosinicum*, a bacterium first isolated from root nodules of *Albizia julibrissin* [18]. These results, however, do not agree with those previously published, in which *R. etli* and *R. gallicum* were isolated from nodules of *P. vulgaris* growing in Egyptian soils [25]. The discrepancy may reflect differences in soil characteristics, since *R. etli* and *R. gallicum* were isolated from plants grown

Table 2. EzTaxon-e closest relative species of strains isolated in this study

Strains	Original host	Closest related type strains	Similarity (%)
BSPV2	*P. vulgaris*	*R. leguminosarum* USDA 2370 T	99.76
BSPV7	*P. vulgaris*	*R. leguminosarum* USDA 2370 T	99.76
BSPS4	*P. sativum*	*R. leguminosarum* USDA 2370 T	99.76
BSVF2	*V. faba*	*R. leguminosarum* USDA 2370T	99.76
BSVF5	*V. faba*	*R. leguminosarum* USDA 2370 T	99.76
BSVF9	*V. faba*	*R. leguminosarum* USDA 2370 T	99.61
BSLE4	*L. esculentus*	*R. leguminosarum* USDA 2370 T	99.61
BSLE10	*L. esculentus*	*R. leguminosarum* USDA 2370 T	99.61
BSPV9	*P. vulgaris*	*R. lusitanum* P1-7 T	100.00
BSPV11	*P. vulgaris*	*R. mesosinicum* CCBAU 25010 T	99.84
BSPS7	*P. sativum*	*R. pisi* DSM 30132 T	99.92
BSPS10	*P. sativum*	*R. etli* CFN 42 T	99.84
BSCA1	*C. arietinum*	*M. amorphae* ACCC 19665 T	100.00
BSCA8	*C. arietinum*	*M. robiniae* CCNWYC 115 T	100.00
BSCA9	*C. arietinum*	*M. robiniae* CCNWYC 115 T	100.00

in desert areas [25] whereas *R. leguminosarum*, *R. lusitanum*, and *R. mesosinicum* were obtained from nodules of plants cultivated in agricultural, fertile areas.

Strains isolated from *P. sativum* were discriminated into three genotypes, *R. leguminosraum*, *R. pisi*, and *R. etli* (Table 2). *Pisum sativum* was previously shown to be nodulated by *R. leguminosarum* and *R. pisi* [10, 25]. The latter species was originally isolated from nodules of pea plants and corresponds to a reclassification of strain *R. leguminosarum* DSM 30132 [21]. To our knowledge, ours is the first report showing that *R. etli* produces effective nodules on roots of *P. sativum*.

Strains from *C. arietinum* were identified as *M. amorphae* and *M. robiniae* (Table 2). *M. amorphae* was isolated from *Amorpha fruticosa* plants grown in Chinese [43] and American soils [44], and *M. robiniae* is found in root nodules of *Robinia pseudoacacia* growing in China [48], but there were no reports that this rhizobial species nodulates *C. arietinum*.

The only species isolated from root nodules of *V. faba* was *R. leguminosarum* (Table 2), which is consistent with previous reports [10,24]. *R. leguminosarum* was shown to form nodules in faba bean plants from Ethiopia [2], France [7], Jordan [20], China [34], and Canada [41]. It was also the predominant rhizobial species isolated from nodules of agriculturally grown faba bean plants in Egypt [27].

Rhizobial strains isolated from *L. esculentus* grouped into two genotypes that were identified as *R. leguminosarum* (Table 1). *R. leguminosarum* bv. viciae is the specific microsymbiont of the legumes of the tribe Vicieae, which comprises the genera *Vicia*, *Pisum*, *Lens*, and *Lathyrus* [10, 24]; accordingly, its isolation from Egyptian lentils is not surprising.

Because inoculation of legumes is a common practice in Egypt [45, 46], the identification, selection, and maintenance of superior rhizobial strains for each host plant are critical. Collectively, based on 16S rRNA gene sequences, our results show that Egyptian agriculturally grown members of the genus *Lens* as well as broad beans, peas, common beans, and chickpeas can be nodulated by different species of *Rhizobium* and *Mesorhizobium*. The recognition of this diversity is essential to improve our knowledge of endosymbiotic bacterial populations and thus to study the activities and applications

of rhizobial strains important in agriculture, environmental protection, and biotechnology. In this context, we isolated a number of rhizobial strains that could be assayed for increased productivity of agriculturally grown legumes in Egypt. The 15 selected strains identified in this study are true symbionts of their corresponding host plant as, after nodule isolation, they were able to establish new and effective N_2-fixing symbioses with them. Although the acetylene reduction technique cannot be used as a quantitative assay of N_2 fixation [36], in this study it allowed us to determine whether the nodulated legume roots actively fixed N_2 but not the effectiveness of each symbiotic association.

Acknowledgements. This study was supported by ERDF-cofinanced grant RNM4746 from Consejería de Economía, Innovación y Ciencia (Junta de Andalucía, Spain). A.M. Mahmoud thanks the Egyptian Government for a Partnership and Ownership Initiative (PAROWN) grant to support his stay in the Department of Microbiology and Symbiotic Systems, Estación Experimental del Zaidín (EEZ), Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain.

Competing interests. None declared

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