Biol Fertil Soils (2004) 39:179–185
DOI 10.1007/s00374-003-0697-z

ORIGINAL PAPER

with Compliments.
Fauzia 8/03/04

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Rhizobium, *Bradyrhizobium* and *Agrobacterium* strains isolated from cultivated legumes

Received: 8 May 2003 / Accepted: 18 November 2003 / Published online: 18 December 2003

Abstract The present study was conducted to isolate and characterize rhizobial strains from root nodules of cultivated legumes, i.e. chickpea, mungbean, pea and siratro. Preliminary characterization of these isolates was done on the basis of plant infectivity test, acetylene reduction assay, C-source utilization, phosphate solubilization, phytohormones and polysaccharide production. The plant infectivity test and acetylene reduction assay showed effective root nodule formation by all the isolates on their respective hosts, except for chickpea isolate Ca-18 that failed to infect its original host. All strains showed homology to a typical *Rhizobium* strain on the basis of growth pattern, C-source utilization and polysaccharide production. The strain Ca-18 was characterized by its phosphate solubilization and indole acetic acid (IAA) production. The genetic relationship of the six rhizobial strains was carried out by random amplified polymorphic DNA (RAPD) including a reference strain of *Bradyrhizobium japonicum* TAL-102. Analysis conducted with 60 primers discriminated between the strains of *Rhizobium* and *Bradyrhizobium* in two different clusters. One of the primers, OPB-5, yielded a unique RAPD pattern for the six strains and well discriminated the non-nodulating chickpea isolate Ca-18 from all the other nodulating rhizobial strains. Isolate Ca-18 showed the least homology of 15% and 18% with *Rhizobium* and *Bradyrhizobium*, respectively, and was probably not a *(Brady)rhizobium* strain. Partial 16S rRNA gene sequence analysis for MN-S, TAL-102 and Ca-18 strains showed 97% homology between MN-S and TAL-102 strains, supporting the view that they were strains of *B. japonicum* species. The non-infective isolate Ca-18 was 67% different from the other two strains and probably was an *Agrobacterium* strain.

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Keywords *(Brady)rhizobium* · *Agrobacterium* · RAPD · 16S rRNA

Introduction

Successful management of symbiotic association between leguminous plants and their bacterial endosymbionts requires the identification of these bacterial strains by easy, rapid and reliable methods (Selenska-Pobell et al. 1995, 1996). Traditional methods used for identifying microbial strains were based on morphological, physiological and biochemical assay (Echeverrigaray et al. 2000). However, these methods frequently failed in the identification of *Rhizobium* strains within a species and there are circumstances in which recognition of a particular strain of *Rhizobium* and the monitoring of its occurrence following introduction to the soil environment is important. Molecular methods that complement the traditional microbiological procedures have been effectively adopted for strain identification (Wu and Tranksley 1993; Liu et al. 1997; Muyzer and Smalla 1998; Muyzer 1999). Little is known about the relationships between genetic polymorphism within a rhizobial population and the preference for nodulating a particular genotype of compatible host plants (Saleena et al. 2001). Among the techniques developed to detect DNA polymorphisms in many different organisms including bacteria by PCR, random amplification of polymorphic DNA (RAPD) is one of the quickest and easiest (Paffetti et al. 1996; Gougeon et al. 2000). This genomic fingerprinting technique has been applied to characterize *Rhizobium* at the level of strains (Bowditch et al. 1993; Paffetti et al. 1996).

Many nitrogen-fixing *Rhizobium* (Terefework et al. 1998) and actinomycete (Hameed et al. 1994; Mirza et al. 1994) species have been effectively characterized on the basis of sequence homology of 16S rRNA. The 16S rRNA stretches of sequence are conserved to varying degrees and their positions are mostly known. Sequence information from the conserved region is useful for studying

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phylogenetic relationships (Woese et al. 1985) as well as for designing universal oligonucleotide probes and primers to be used for identification and amplification, respectively (Hameed et al. 1994).

The main aim of this study was to characterize six bacterial isolates from root nodules of pea, chickpea, siratro, mungbean and soybean with traditional methods based on their morphology, ability to fix dinitrogen, to produce phytohormones like IAA, to solubilize phosphate and their ability to grow on different C-sources. These methods were combined with RAPD analysis so as to identify the relative species and particularly the polymorphism among *Rhizobium* strains. The sequence of 16S rRNA of some isolates was also analyzed to assess taxonomical affinity. We hope that this work will help to identify potentially beneficial bacterial isolates for biofertilizer-aided production of various economically important crops.

Materials and methods

Morphological, physiological and biochemical characterization

The bacterial strains used in this study are listed in Table 1. The bacterial strains were grown on yeast extract mannitol (YEM) agar (Vincent 1970), supplemented with bromothymol blue and Congo red as an indicator (Nelson and Child 1981), with shaking at 28±2°C and studied for mean generation time and Gram's reaction (Vincent 1970). The morphological and cultural characteristics of the bacterial strains were studied by light microscopy.

Host specificity and effectiveness of the *Rhizobium* strains were studied on *Cicer arietinum*, *Glycine max*, *Macroptium atropurpureum* cv. *siratro*, *Pisum sativum* and *Vigna radiata*. Seeds were obtained from the Mutation Breeding Division of NIAB, Faisalabad, Pakistan. Seeds were surface-sterilized with 0.1% $HgCl_2$, germinated on water agar plates and grown in growth pouches under controlled environmental conditions. Inoculum of 1 ml each strain was applied to the roots of the respective legume. There were three replicates for each strain with a negative control. Plants were harvested 30 days after sowing and nitrogenase activity of the nodules was determined by acetylene reduction assay (Hardy et al. 1968).

Strains were grown on combined C-medium (Rennie 1981) containing different sugars as C sources: L-arabinose, maltose, galactose, glucose, rafinose, mannitol, molasses, sucrose and xylose. All strains were streaked in triplicate and incubated at 28±2°C. The presence or absence of growth was observed after 3- to 5-days incubation.

A single colony from each bacterial culture grown on YEM medium was streaked on Pikovskaia's medium containing tricalcium phosphate (Pikovskaia 1948) and incubated at 28±2°C for 7–10 days. The plates were observed for clear P-zone formation around colonies. Quantification of solubilized phosphates was carried out by the phospho-molybdate method using a spectrophotometer (Yoshida et al. 1976).

For detection and quantification of IAA production by the bacterial isolates, cultures were grown in Okon's malate medium (Okon et al. 1977). As a precursor of IAA, 100 mg l^{-1} tryptophan was added. After 1 week of growth, qualitative estimation of IAA was performed using $FeHClO_4$ and FeH_2SO_4 reagents (Gordon and Weber 1951). For quantitative estimation of IAA by HPLC, the ethyl acetate oxidation method was used (Tien et al. 1979). The samples were analyzed on HPLC using Turbochem software (Perkin Elmer, United States).

Total hydrolysable sugars of the bacterial cells were determined quantitatively by hydrolysis of polysaccharide fractions. Reducing sugars were estimated spectrophotometrically using dinitro salicylic acid reagent (DNS; Miller 1959).

RAPD analysis and 16S rRNA sequencing

Bacterial strains were grown in YEM broth to log phase and centrifuged at 6,000x *g* for 10 min at 4±1°C. The total genomic DNA was extracted from the different bacterial strains (Sambrook et al. 1989) and quantified by fluorometer (Hoefer DyNA Quant TM200, San Francisco, Calif.).

The PCR procedure was followed as described by Williams et al. (1990). Out of the 60 random 10-mer primers (Operon Technologies, United States) used, 15 were from the OPA series, 10 from the OPB series, 15 from the OPC series, 10 from the OPJ series and 10 from the OPR series. The reaction volume was 25 µl,

Table 1 Host, source and physiological characteristics of the *Rhizobium* and *Bradyrhizobium* strains isolated from leguminous plants (*ARA* acetylene reduction assay, *P* phosphate solubilization, *IAA* indole acetic acid)

Strain	Species	Host	Source	Acid/Alkali production	ARA	P (µg ml^{-1})	IAA[a]	IAA[b] (µg ml^{-1})	Total hydrolyzable sugars (mg ml^{-1} glucose)
MN-S	*Bradyrhizobium* sp.	Mungbean[c], soybean	Pakistan	Alkali	+	-	+	0.04	3.6
TAL-102	*B. japonicum* [d]	Soybean[c], mungbean	USA	Alkali	+	-	+	ND	3.2
Ca-18	Isolate to be identified	Chickpea[c]	Pakistan	Acid	-	63.3	+	35	5.8
PS-1	*Rhizobium leguminosarum* bv. *viciae*	Pea[c], lentil	Pakistan	Acid	+	-	+	0.34	5.5
PS-2	*Rhizobium leguminosarum* bv. *viciae*	Pea[c], lentil	Pakistan	Acid	+	-	+	ND	5.6
Ma-8	*Rhizobium* sp.	Siratro[c], mungbean	Pakistan	Acid	+	-	+	ND	5.3

[a] Colorimetric method
[b] HPLC
[c] Authenticated host
[d] Reference strain

containing 3 μl (25 mM) $MgCl_2$, 4 μl (2.5 μM) dNTPs, 2 μl (15 ng μl^{-1}) primer, 2 μl (12.5 ng μl^{-1}) genomic DNA, 2.5 μl (0.025%, w/v) gelatin, 2.5 μl 10× PCR buffer and 0.2 μl (1 unit) *Taq* polymerase. *Taq* polymerase, 10× PCR buffer, $MgCl_2$, dNTPs and gelatin were purchased from Sigma Chemicals. Amplification was carried out in a Perkin Elmer DNA Thermal Cycler 480 programmed for a first denaturation step of 5 min followed by 40 cycles of 1 min at 94°C, 36°C for 1 min and 2 min at 72°C. Finally the reaction mixture was kept at 72°C for 10 min. Amplification products were analyzed by electrophoresis in 1.2% (w/v) agarose gels and detected by staining gels with ethidium bromide (10 ng 100 ml^{-1} agarose solution in TBE).

All visible fragments amplified by primers were scored under the heading of total scorable fragments. Amplification profiles of the six rhizobial isolates were compared with each other and PCR fingerprints were converted to a two dimensional binary matrix (1, presence of a PCR product; 0, absence of a PCR product). The RAPD results were a consensus of at least two replications. Average linkage (UPGMA—unweighted pair group method of averages) was based on similarity coefficients (Nei and Li 1979).

The total genomic bacterial DNA of MN-S, TAL-102 and Ca-18 was isolated by the alkaline lysis method (Maniatis et al. 1982). The primers used for PCR amplification and sequencing were universal primers Y1 and Y2, as previously used for proteobacteria by Young et al. (1985) and for acidic soil rhizobia (Del Papa et al. 1999). The forward primer Y1 (5′-TGGCTCAGAACGAACGCTGGCGGC-3′) corresponds to positions 20–43 in the *Escherichia coli* 16S rRNA sequence (Young et al. 1991), and the reverse primer Y2 (5′-CCCACTGCTGCCTCCCGTAGGAGT-3′) corresponds to positions 361–338 of the gene.

Amplification of the partial 16S rRNA gene was carried out using DNA isolated from the three strains as template. Each 50 μl reaction mixture contained 0.2 μl Taq Polymerase (50 U μl^{-1}; HT Biotechnology, United Kingdom), 5 μl 10× buffer, and 4 μl dNTPs with a final concentration of 2.5 mM of each, 1 μl (100 ng μl^{-1}) each primer, and 1 μl nucleic acid preparation with a final concentration of 12.5 ng μl^{-1}, and underwent 35 rounds of temperature cycling (94°C for 1 min, 50°C for 2 min and 72°C for 3 min). This was followed by incubation at 72°C for 7 min.

The amplification products were confirmed on 1.2% TAE agarose gel, followed by excision of the fragments and subsequent purification by Gene-Clean procedure using the CONCERT Rapid Gel Extraction System, GIBCO/BRL. Cloning of the amplification products into PCR 2.1 vector was done by adapting standard methods (Maniatis et al. 1982) using the PCR 2.1 cloning kit, and then they were transformed into $CaCl_2$-mediated *E. coli* competent cells. Plasmids were isolated for sequencing following the Mediprep protocol using the CONCERT Rapid Isolation System Kit, GIBCO/ BRL, and nucleotide sequences of the partial 16S rRNA gene were determined for both strands. Cloned PCR products were sequenced using the Diprimer Cycle Sequencing Kit with M13 forward and reverse primers and the Big Dye Primer Kit in a Perkin Elmer ABI Prism 310 genetic analyzer. After sequence comparison, the results were searched on Blast x (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The 16S rRNA sequences of tested strains in this study were submitted to the EMBL GenBank database for their confirmation and classification.

Results and discussion

Morphological, biochemical and physiological characterizations

All rhizobial strains were identified on the basis of morphological, biochemical and physiological characteristics (Table 1). The colonies produced were gummy, translucent, circular and convex with entire or smooth margins. They showed a mean generation time of 24–72 h. The Gram staining technique showed that all the strains were Gram-negative and rod-shaped under the light microscope.

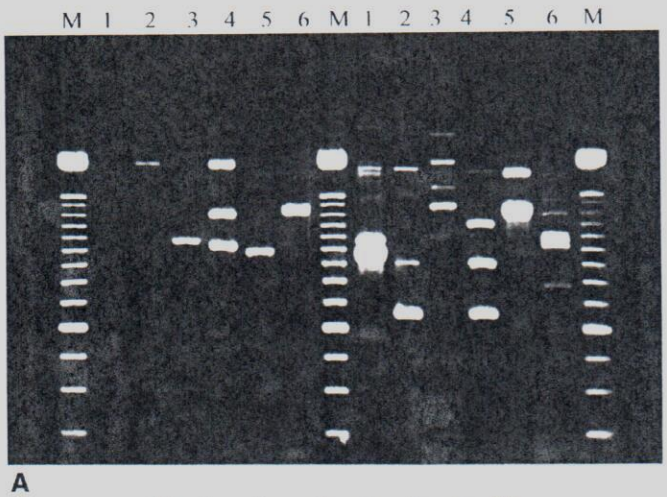
The rhizobial strains PS-1, PS-2, Ma-8 and Ca-18 showed sufficient growth in 3 days and turned the YEM agar medium with bromo thymol blue to yellow showing that these were fast growing, acid producers having a mean generation time of 24 h. On the contrary, the strains TAL-102 and MN-S were slow growing and produced a blue coloration of the medium showing that these were alkali producers having a mean generation time of 48–72 h (Keyser et al. 1982; Anand and Dogra 1991). All six strains were equally efficient in utilizing L-arabinose, maltose, galactose, glucose, rafinose, mannitol, molasses, sucrose and xylose. Therefore, the utilization of different C-sources could not discriminate the fast growing and slow growing strains. Similar findings have been reported by Hafeez et al. (1995). The plant infectivity test and ARA discriminated new chickpea isolate Ca-18 from other effective nodule-forming strains of *Bradyrhizobium* (MN-S and TAL-102) and *Rhizobium* (PS-1, PS2, Ma-8; Table 1). It is evident from previous data that a wide range of strains ranging from fast- to slow- and even very-slow-growing may form effective nodules. Occupancy of nodules by Ca-18 is still important as there is evidence that multiplication within a nodule is important for maintaining soil populations of ineffective rhizobia (Sprent 1994).

The results showed that the strains MN-S, TAL-102, PS-1, PS-2 and Ma-8 did not have an ability to solubilize P. The Ca-18 strain was the only one which formed colonies with clear zones around them indicating its ability to solubilize P (Table 1); it showed 63.3 $\mu g\ ml^{-1}$ P-solubilization, with an increase of 155% over the uninoculated control. Therefore, Ca-18 is a novel strain regarding its importance as a strong P-solubilizer which could be used in multistrain inocula for biofertilizer production (Bashan and Holguin 1997). All the strains under study were able to produce IAA (Table 1).

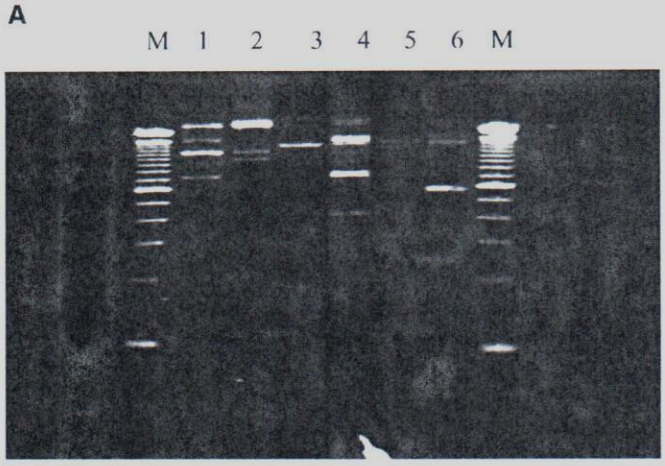
The results showed that strains Ca-18, PS-1, PS-2 and Ma-8 produced greater amounts of exopolysaccharides in the medium while MN-S and TAL-102 were found to be poorer gum producers. Total amounts of hydrolysable sugars detected differentiated fast-growing rhizobial strains from slow-growing strains of *Bradyrhizobium* and placed Ca-18 among the rhizobial strains. On the basis of physiological tests, we can conclude that most of the locally isolated strains (Ca-18, PS-1, PS-2 and Ma-8) are fast growing and are high exopolysaccharide producers (Sardar 2000).

Random amplified polymorphic DNA analysis

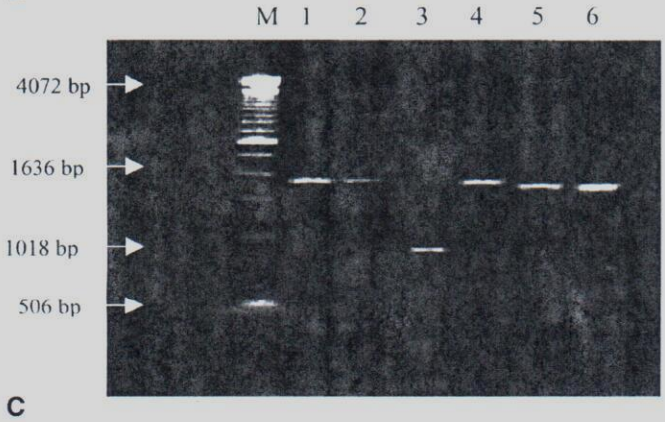
RAPD was used to study polymorphism among the six (*Brady*) *rhizobium* strains and to investigate the extent of homology of Ca-18 with the strains of *Rhizobium* and *Bradyrhizobium*. It is important to know the phylogenetic



A



B



C

Fig. 1A–C Random amplified polymorphic DNA patterns of six *Rhizobium* and *Bradyrhizobium* strains isolated from different legumes. Lane/strains: *M* 1 kb ladder, *1* MN-S, *2* TAL-102, *3* Ca-18, *4* PS-1, *5* PS-2, *6* Ma-8. **A** Primer OPC-14 and OPC-15. **B** Primer OPC-13. **C** Primer OPB-5

systematics of Ca-18 as it was isolated from a nodule of *C. arietinum* but did not form nodules when reinoculated on the same host. Moreover, it is an effective strain regarding its P-solubilizing ability and phytohormone production.

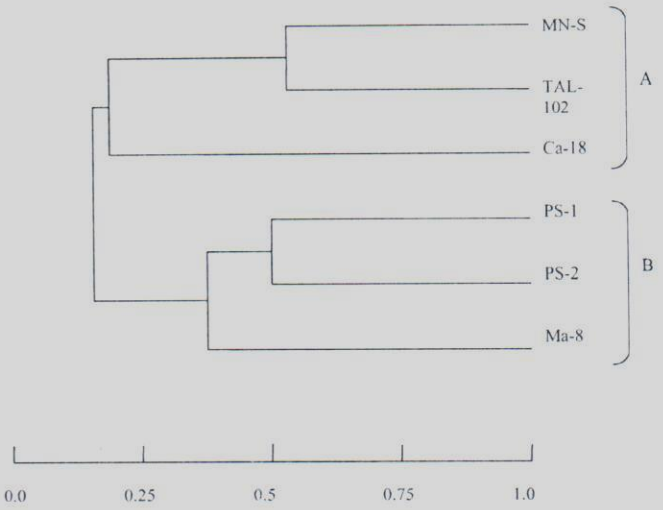


Fig. 2 Dendrogram showing the genetic relationship of six *Rhizobium* and *Bradyrhizobium* strains isolated from different legumes as determined by RAPD analysis

RAPD analysis was performed with 60 decamer oligonucleotide primers. Random priming amplification showed polymorphism among all the six strains and differentiated between the strains of *Rhizobium* and *Bradyrhizobium*. A total of 790 DNA fragments were amplified, with an average of approximately 13 bands per primer. From these, 99.8% were polymorphic and 0.2% monomorphic. Primer OPC-15 produced the greatest number of bands. The fragments amplified were in the range of 0.3 to 3.5 kb (Fig. 1A–C). The genetic similarities ranged from 15% to 52% (Fig. 2). Moreover, a dendrogram showed that the strains were clustered into two groups branching at a similarity of 15%. The similarity among the isolates of group A, formed by the three strains MN-S, TAL-102 and Ca-18, ranged from 18% to 52%. Group B consisted of the three strains PS-1, PS-2 and Ma-8 with similarities ranging from 38% to 50% (Fig. 2). The close genetic relationship between MN-S and TAL-102 is due to the fact that they are strains of *Bradyrhizobium* sp. (cowpea misc.) and *B. japonicum*, respectively, and also showed nodulation when cross inoculated to the hosts of one another. The greater similarity between strains PS-1 and PS-2 compared to Ma-8 is evident from the fact that the first two are strains of *R. leguminosarum* bv. *viciae*, forming nodules on the same host (pea). A higher level of polymorphism was observed between the strains of *Rhizobium* and *Bradyrhizobium* as compared to strains belonging to the same genus of *Rhizobium/Bradyrhizobium*. These findings are well supported by previous data showing that *Rhizobium* and *Bradyrhizobium* are only distantly related (Martinez-Romero 1994).

Ca-18 was the least similar isolate as it was genetically 18% similar to the strains of *Bradyrhizobium* MN-S and TAL-102 and was 15% similar to group B comprising the strains of *Rhizobium*. Comparatively less polymorphism between fast-growing strain Ca-18 and slow-growing

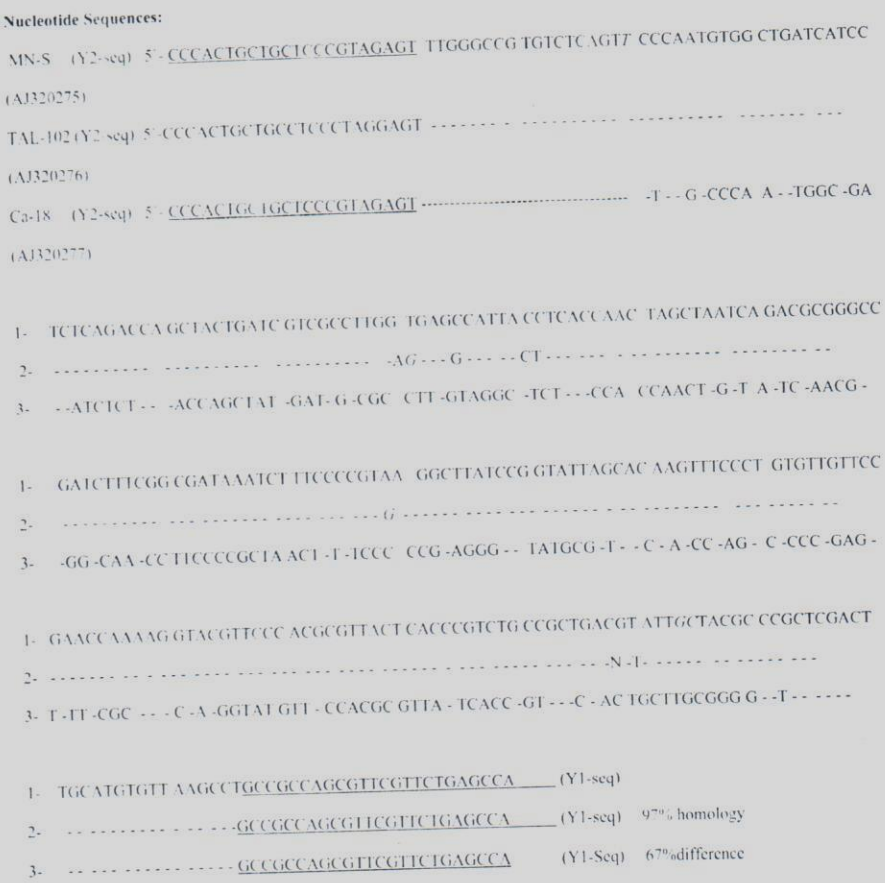


Fig. 3 Alignment of sequences from the variable regions of the 16S rRNA amplified by PCR from MN-S, TAL-102 and Ca-18. The target sequences for primers Y1 and Y2 are *underlined*. Matches in the sequences are shown as *a dash*

strains of *Bradyrhizobium* can be justified from the fact that the fast-growing strains are very diverse and some can be even closer to *Bradyrhizobium* than *Rhizobium* (Hollis et al. 1981; Stanley et al. 1985; Barnet et al. 1993). The low similarity of Ca-18 with other nodule-forming strains can be justified by the fact that although it was isolated from nodules of *C. arietinum*, it did not form nodules when reinoculated on the same host. Growth pattern, cell and colony morphology, acidification of YEM containing bromothymol blue and its repeated isolation from nodules of chickpea confirmed that the isolated strain Ca-18 was *Rhizobium* sp., but RAPD results showed that genetically it is quite different from *Rhizobium*. One of the primers, OPB-5, also yielded a unique RAPD pattern for the six strains and well discriminated Ca-18 from all the other strains, demonstrating the utility of this technique for distinguishing between *Rhizobium* and *Bradyrhizobium* isolates (Fig. 1C). Even though strain Ca-18 was found to be a non-nodulating strain and did not fix N, as indicated by the plant infectivity test and acetylene reduction assay, we cannot assume that it is a poor-quality symbiont that does not benefit the host plant; it seemed to be a competitive strain that replaced other bacteria and may play an important role as a companion strain of effective rhizobia. Moreover, Ca-18 is a novel strain regarding its importance as an effective P-solubilizer and growth hormone producer and can be recommended as an inoculant strain for biofertilizer production. Since these studies only provided information about the genetic relationship of rhizobial strains and provided preliminary evidence that Ca-18 does not belong to (*Brady*)*rhizobium* sp., further studies were carried out to confirm the phylogenetic status of Ca-18 using 16S rRNA gene sequence analysis, and to ascertain the importance of *Rhizobium*-like soil bacteria lacking symbiotic information.

Analysis of 16S rRNA sequences

Through 16S rRNA sequence analysis it was possible to differentiate among morphologically and physiologically similar bacterial strains due to differences within a partial but conserved region of the gene. The reported partial 16S rRNA sequence of *B. japonicum* reference strain TAL-102 (accession no. AJ320276) significantly aligned with 25 different species of *B. japonicum*, with a 494 score bit and E value of 137. The sequence reported for strain MN-S (accession no. AJ320275) produced significant alignments with 20 different species of *B. japonicum* with a 494 score bit and E value of 137 confirming that MN-S may be a strain of *B. japonicum*. The sequence reported for the plant growth-promoting strain Ca-18 (accession no. AJ320277) produced significant alignments with 25 different species of *Agrobacterium*, out of which 6 species had a 369 score bit and E value of 100, and out of these 6

species 2 were *A. tumefaciens*. On the basis of GenBank database study Ca-18 may be a strain of *Agrobacterium* sp. It was also confirmed that all the three strains belong to the family Rhizobiaceae, class Proteobacteria.

The sequence analysis results revealed that the two strains MN-S and TAL-102 are closely related with a sequence homology of 97% (Fig. 3) and are strains of *B. japonicum*. This is also indicated by their cell morphology, growth pattern, and other biochemical aspects (Table 1), as well as RAPD profile (Fig. 2). The third strain, Ca-18, which is morphologically similar to MN-S and TAL-102, was a chickpea isolate, but failed to reinfect its host. Moreover, Ca-18 had shown 67% dissimilarity and only 33% homology with the two *B. japonicum* strains and was found to be an *Agrobacterium* sp. (Fig. 3).

To conclude, both RAPD profile and partial 16S rRNA sequence analysis seem to indicate that Ca-18 is a strain of *Agrobacterium*; this strain can occupy nodule cells and may have the potential benefit of being able to solubilize phosphate. This bacterial isolate can be effectively utilized as a biofertilizer for various crops.

Acknowledgements The research work was funded partly by the IDB BIRCEN project and FAO/IAEA TC project no. PAK/5/037. We are thankful to Ms. Fathia Mubeen and Mr. Mazhar Hussain for their help in DNA sequencing work, as well as to Dr. Farooq Latif for his help with HPLC to detect phytohormones.

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