A novel bacterial strain, designated NRCPB10T, was isolated from rhizosphere soil of chickpea (Cicer arietinum L.) in Pusa, New Delhi, India. The 16S rRNA gene sequence of strain NRCPB10T showed highest similarity (98.9%) to that of Rhizobium radiobacter NCPPB 2437T, followed by Rhizobium larrymoorei AF3-10T (97.7%) and Rhizobium rubi IFO 13261T (97.4%). Phylogenetic analysis of strain NRCPB10T based on the housekeeping genes recA and atpD confirmed its position as distinct from recognized Rhizobium species. Levels of DNA–DNA relatedness between strain NRCPB10T and R. radiobacter ICMP 5785T, R. larrymoorei LMG 21410T and R. rubi ICMP 6428T were 51.0, 32.6 and 27.3%, respectively. Cellular fatty acids of strain NRCPB10T were C18:1ω7c (58.9%), C16:0 (15.5%), C19:0 cyclo ω8c (11.5%), iso-C16:1 (5.8%), C16:0 3-OH (4.5%), C18:1ω7c (2.1%) and C18:0 (1.3%). The G+C content of the genomic DNA of strain NRCPB10T was 59.0 mol%. Strain NRCPB10T did not nodulate chickpea plants or induce tumors in tobacco plants. Phenotypic and physiological properties along with SDS-PAGE of whole-cell soluble proteins differentiated strain NRCPB10T from its closest phylogenetic neighbours. On the basis of data from the present polyphasic taxonomic study, strain NRCPB10T is considered to represent a novel species of the genus Rhizobium, for which the name Rhizobium pusense sp. nov. is proposed. The type strain is NRCPB10T (=LMG 25623T =JCM 16209T =NCIMB 14639T).

Rhizobium species were originally known for their symbiotic association with members of the leguminosae, and their nomenclature was shaped by the specificity of their symbiotic plant range. However, symbiotic properties in some rhizobia are genetically unstable, raising the possibility that non-symbiotic rhizobia are a significant component of rhizobial populations in the soil (Sullivan et al., 1996). The occurrence of non-symbiotic rhizobia in the environment has not been extensively investigated, because of the difficulty of isolating rhizobia directly from the soil or rhizosphere (Laguerre et al., 1993; Segovia et al., 1991). However, some free-living strains of Rhizobium species have been reported from soil (Soberon-Chávez & Najera, 1989; Quigley et al., 1997), rhizosphere and plant roots (Segovia et al., 1991; Yanni et al., 1997; Rosenbluth & Martinez-Romero, 2004). These non-symbiotic strains may have arisen through loss of symbiotic plasmids, either before or during their isolation and subsequent culture (Laguerre et al., 1993). In addition, several Rhizobium species have been described on the basis of non-symbiotic strains isolated from bioreactors (Quan et al., 2005; Hunter et al., 2007). Therefore, the non-symbiotic lifestyle of rhizobia is likely to be involved in a broad range of functions in diverse ecosystems.

This paper describes a novel bacterial strain, designated NRCPB10T, isolated from the rhizosphere of a chickpea plant. Based on the results of our polyphasic taxonomic study, strain NRCPB10T is considered to represent a novel species of the genus Rhizobium.

Strain NRCPB10T was isolated from the rhizosphere soil of chickpea (Cicer arietinum L.) grown in Pusa, New Delhi, northern India. Roots with adhering soils were collected 2 months after planting. For sampling, plants were uprooted by hand, shaken to dislodge loose soil and the roots were excised. To isolate bacteria, roots were submerged in 25 ml washing buffer (0.1 M phosphate buffer, pH 7.0) with slow stirring. Serial dilutions of the samples in distilled water were plated onto yeast–mannitol (YM) agar medium (Vincent, 1970). The plates were incubated for 4 days at 28°C. Several colonies that developed at 28°C were picked and purified by repeated streaking on the same medium. A colony with a
mucoid cream–white colour was picked as being representative of several similar colonies, designated strain NRCPB10T and studied further. Media were sterilized by autoclaving at 103 kPa for 15 min prior to use. For preparation of solid media, 15 % agar–agar (Difco) was used.

Cell morphology was examined by using transmission electron microscopy (Morgagni 268D; FEI). The presence of flagella was determined by using cells grown on YM agar for 2 days. Cells were placed on to a carbon-coated grid and negatively stained with 2.0 % phosphotungstic acid (Sharma et al., 1989). Colonies of strain NRCPB10T grown on YM agar were circular, 2.0–3.0 mm in diameter, mucoid and smooth after incubation for 3 days at 28 °C. Cells occurred mostly singly, were rod-shaped, 0.5–0.6 μm wide and 1.2–1.5 μm long and were motile by means of a single polar flagellum (Supplementary Fig. S1 in IJSEM Online). Cells stained Gram-negative. Oxidase activity was assayed with discs impregnated with dimethyl p-phenylenediamine (Hi-Media). Catalase activity was assayed by mixing a pellet of a freshly centrifuged culture with a drop of hydrogen peroxide (10 %, v/v). Biochemical characteristics were studied by using the Biolog GN2 (Biolog) and Hi25™ (Hi-Media) identification kits as recommended by the manufacturers. Anaerobic growth was determined with a BD GasPak EZ system (Becton Dickinson) as per the manufacturer’s instructions. Bacterial growth was monitored by measuring OD600 in a Specord210 spectrophotometer (Analytik).

Phenotypic properties of strain NRCPB10T are given in the species description below and characteristics that differentiate it from closely related species are detailed in Table 1. The pH range for growth was determined in YM medium in the range pH 4.0–12.0 in steps of 0.5 pH units. pH was adjusted by addition of 1 M HCl or 1 M NaOH. Medium was sterilized by membrane filtration (pore size, 0.22 μm; Millipore). Growth was observed at pH 5.0–11.0, with optimum growth at pH 7.5. The temperature range for growth was tested in YM broth at 15–45 °C. Strain NRCPB10T was able to grow at 16–41 °C, but not at 15 or 42 °C. The optimum growth-temperature range was 28–30 °C. The NaCl range for growth was examined in YM broth containing 0–5.0 % (w/v) NaCl at 28 °C for 4 days. Strain NRCPB10T was able to grow in the presence of 0–4 % NaCl, but not with 5 % NaCl. Antibiotic resistance was determined on YM agar medium supplemented with different antibiotics. Strain NRCPB10T was resistant to (µg ml⁻¹) ampicillin (50), nalidixic acid (50) and trimethoprim (50), but was susceptible to tetracycline (20), rifampicin (25), neomycin (50), kanamycin (30), streptomycin (50) and spectinomycin (50).

The G+C content of the purified DNA was determined by HPLC (Shimadzu) as described by Mesbah et al. (1989).

Table 1. Differential characteristics between strain NRCPB10T and the type strains of related *Rhizobium* species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth with 4 % NaCl</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Carbon-source utilization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrin, inosine</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glycogen, L-arabinose, lactulose, DL-lactic acid</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tween 40, Tween 80, cis-aconitic acid, D-galacturonic acid, β-hydroxybutyric acid, propionic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetyl-D-galactosamine, N-acetyl-D-glucosamine, α-hydroxybutyric acid, α-ketobutyric acid</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-Lactose, 1-pyrogulatamic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Succinic acid monomethyl ester, D-glucaminic acid, citric acid, p-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-leucine, γ-aminobutyric acid, 2,3-butanediol, phenylethylamine, thymidine, DL-carnitine, urocanic acid, l-erythritol, l-phenylalanine</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Acetic acid, D-galactonic acid lactone, D-gluconic acid, D-alanine, glycyll L-aspartic acid, hydroxy-L-proline</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Glucurononic acid, raffinose, formic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Succinic acid, bromosuccinic acid</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycyl L-glutamic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Serine, uridine, L-histidine, L-alamyl glycine, quinic acid, D-saccharic acid, L-rhamnose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Serine, L-aspartic acid</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>α-D-Glucose 1-phosphate, D-glucose 6-phosphate, glyceral</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-alanine, myo-inositol, gentiobiose, melibiose, D-psicose, xylitol</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>
The DNA G+C content of strain NRCPB10T was 59.0 mol%, which is within the range reported for the genus *Rhizobium* (57–66 mol%; Young et al. 2001).

Cellular fatty acids were analysed after growing strain NRCPB10T and its close relatives *Rhizobium radiobacter* ICMP 5785T, *Rhizobium larrymoorei* LMG 21410T and *Rhizobium rubi* ICMP 6428T on YM agar plates at 28 °C for 3 days. Cells were saponified and transmethylated as described by Kuykendall et al. (1988). The fatty acid methyl ester mixtures were separated by using the Sherlock Microbial Identification System (library, RTSBa6 6.00; MIDI) and an Agilent model 6890N gas chromatograph. The cellular fatty acid profile of strain NRCPB10T comprised C\textsubscript{18:1}ω7c (58.9 %), C\textsubscript{16:0} (15.5 %), C\textsubscript{19:0} cyclo ω8c (11.5 %), iso-C\textsubscript{16:1} (5.8 %), C\textsubscript{16:0} 3-OH (4.5 %), C\textsubscript{16:1}ω7c (2.1 %) and C\textsubscript{18:0} (1.3 %). This profile of fatty acids matched those of the reference strains. However, strain NRCPB10T could be differentiated based on the absence of C\textsubscript{13:0} and C\textsubscript{17:0} cyclo (Supplementary Table S1).

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene were carried out as described by Das et al. (1996) and Panda et al. (2009). The gel-purified 16S rRNA gene was sequenced by using a CEQ Dye Terminator cycle sequencing kit in a model CEQ 8000 automated DNA sequencer (Panday & Das, 2010). The nucleotide sequences obtained were assembled using the sequence alignment editor program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Searches with the BLAST program for homologous sequences of 16S rRNA genes in the public databases were performed via the NCBI website (Altschul et al., 1997). A neighbour-joining phylogenetic tree was constructed based on 16S rRNA gene sequences according to Kimura’s two-parameter model (Kimura, 1980) by using the MEGA software package, version 4.0 (Tamura et al., 2007). Phylogenetic trees were also constructed with the neighbour-joining method of Saitou & Nei (1987) and with the maximum-likelihood parameter model (Kimura, 1980) by using the MEGA software package. Neighbour-joining trees were also constructed with the neighbour-joining method of Felsenstein, 1985. A similar topology was obtained in the neighbour-joining and maximum-likelihood trees. Therefore, only the neighbour-joining tree is represented in Fig. 1. Altogether, 1379 nt of the 16S rRNA gene was sequenced from strain NRCPB10T and compared with those of closely related strains retrieved from GenBank. Strain NRCPB10T showed 98.9 % 16S rRNA gene sequence similarity to *R. radiobacter* NCPPB 2437T, 97.7 % to *R. larrymoorei* AF3-10T and 97.4 % to *R. rubi* IFO 13261T. In the neighbour-joining tree based on 16S rRNA gene sequences, strain NRCPB10T clustered with the type strain of *R. radiobacter* (Fig. 1).

The housekeeping genes recA and atpD are being used to investigate bacterial phylogeny additional to that based on 16S rRNA gene sequence analysis (Gaunt et al., 2001; Vinuesa et al., 2005; Velázquez et al., 2010). In this study, PCR amplification and sequencing of partial recA (543 bp) and atpD (450 bp) genes of strain NRCPB10T were undertaken according to Gaunt et al. (2001). Levels of recA and atpD gene sequence similarity between strain NRCPB10T and the type strains of reference *Rhizobium* species are given in Table 2. Phylogenetic trees based on recA (Fig. 2) and atpD (Fig. 3) gene sequences were constructed by using the neighbour-joining method of Saitou & Nei (1987). Strain NRCPB10T exhibited levels of recA and atpD gene sequence similarity of 93.8–86.8 and 94.3–89.6 %, respectively, to the type strains of *Rhizobium* species used in this study.

In summary, phylogenetic analysis based on DNA recombinase A (recA) and ATP synthase β (atpD) gene sequences, in agreement with 16S rRNA gene sequence phylogeny, demonstrated the close relationship among strain NRCPB10T, *R. radiobacter*, *R. larrymoorei* and *R. rubi*. However, the position of these taxa relative to other *Rhizobium* species varied in the three phylogenetic trees, suggesting that the 16S RNA gene and housekeeping genes might have had different phylogenetic histories.

DNA–DNA hybridization is considered to be a standard method for defining bacterial species (Wayne et al., 1987). Hence, DNA–DNA hybridization experiments were carried out between strain NRCPB10T, *R. radiobacter* ICMP 5785T, *R. larrymoorei* LMG 21410T and *R. rubi* ICMP 6428T. DNA (1 µg) of each strain was transferred on to a positively charged nylon membrane (Hybond-N+; Amersham) by using a dot-blot apparatus (Bio-Rad). The membrane was air-dried, cross-linked and the DNA probe for each strain was labelled with [α\textsuperscript{32}P]ATP (BRIT) by using a NEBlot-kit (New England Biolabs). Hybridization was performed as described by Ezaki et al. (1989) and Bhadra et al. (2008). Experiments were performed in triplicate with DNA from the type strains of *R. radiobacter*, *R. larrymoorei* and *R. rubi* as probe. Using a labelled probe of strain NRCPB10T, mean DNA–DNA reassociation values were 51 % with *R. radiobacter* ICMP 5785T, 32 % with *R. larrymoorei* LMG 21410T and 27.3 % with *R. rubi* ICMP 6428T (Table 2). Therefore, given the recommended DNA–DNA relatedness cut-off point for species delineation of 70 % (Wayne et al., 1991; Stackebrandt & Goebel, 1994), strain NRCPB10T should be regarded as representing a novel species of the genus *Rhizobium*.

SDS-PAGE of whole-cell soluble proteins has been used widely in rhizobial taxonomy and is suitable for grouping strains at the species level (Diouf et al., 2000; Tan et al., 1997). In this study, SDS-PAGE was performed by using the method of Laemmli (1970) with minor modifications. All cultures were grown in YM broth (Vincent, 1970) at 28 °C for 60 h. Protein samples were prepared by heating 50 mg (wet weight) of cells at 100 °C for 2.0 min in 1.0 ml sample treatment buffer (Laemmli, 1970). The resulting extracts were centrifuged at 10000 g for 15 min. Electropherograms were developed in a Protean II xi vertical electrophoresis cell (Bio-Rad) at 8 mA and 10 °C for 12 h. The protein banding pattern was visualized by staining with 0.25 % Coomassie brilliant blue R-250 in 50 % (v/v) acetic acid.
Fig. 1. Neighbour-joining tree showing the phylogenetic relationship between strain NRCPB10T and the type strains of related species, based on 16S rRNA gene sequences (1379 nt). Bootstrap values based on 1000 resamplings are shown at branch points. Bar, 0.005 substitutions per nucleotide position.
methanol and 10 % acetic acid. Protein patterns were grouped by using the Fingerprinting II software package v. 3.0 (Bio-Rad) developed by Applied Maths, under normalized densitometry. Levels of similarity between the strains were calculated by using the Dice coefficient, and a UPGMA dendrogram was constructed as described by Vauterin & Vauterin (1992). The protein patterns of strain NRCPB10T were different from those of its closest related reference strains (Supplementary Fig. S2). Based on this dendrogram, the level of protein similarity between strain NRCPB10T and \emph{R. radiobacter} ICMP 5785T was 93 %.

Symbiotic properties are important features of rhizobia, and hence the presence of symbiotic genes in strain NRCPB10T was examined by attempting to amplify the \emph{nodA} (Haukka 	extit{et al.}, 1998) and \emph{nifH} (Eardly 	extit{et al.}, 1992; Table 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene sequence similarity (%)</th>
<th>DNA–DNA relatedness (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16S rRNA</td>
<td>recA</td>
</tr>
<tr>
<td>NRCPB10\textsuperscript{T}</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>\emph{R. radiobacter} ICMP 5785\textsuperscript{T}</td>
<td>98.9</td>
<td>93.8</td>
</tr>
<tr>
<td>\emph{R. larrymoorei} LMG 21410\textsuperscript{T}</td>
<td>97.7</td>
<td>88.8</td>
</tr>
<tr>
<td>\emph{R. rubi} ICMP 6428\textsuperscript{T}</td>
<td>97.4</td>
<td>86.8</td>
</tr>
</tbody>
</table>

\textbf{Fig. 2.} Neighbour-joining phylogenetic tree showing the relationship between strain NRCPB10\textsuperscript{T} and the type strains of related species, based on recA gene sequences. Bootstrap values based on 1000 resamplings are shown at branch points. Bar, 0.02 substitutions per nucleotide position.
Poly et al., 2001) genes. However, these genes could not be detected in strain NRCPB10T.

To test the nodulation ability of strain NRCPB10T, seeds of chickpea (Cicer arietinum L.) cv. Pusa C-235 were germinated as described previously (Das et al., 2006) and were planted axenically in Leonard jar assemblies (three seeds per jar) (Leonard, 1943) containing a vermiculite/quartz sand mixture (1:1) and moistened with nutrient medium (McKnight, 1949). Each Leonard jar received 100 ml nutrient medium containing 10^8 bacteria ml^{-1}. To keep the vermiculite/quartz sand mixture moist, 150 ml nitrogen-free nutrient medium was used and the plants were then watered as necessary. Four replicates were considered for each test. Plants were grown in a controlled growth chamber with a 12 h photoperiod and a light intensity of 250 μEm^{-2}s^{-1}. The day temperature was 23 °C, the night temperature was 15 °C and the relative humidity was 60%.

Wild-type NRCPB10T was also inoculated in tobacco plants (Nicotiana tabacum) to check for the formation of tumours. Strain NRCPB10T did not induce nodules in chickpea plants or induce tumours in tobacco plants, suggesting that it is a non-nodulating, non-tumorigenic rhizobium.

In conclusion, the data from the present polyphasic taxonomic study demonstrate that strain NRCPB10T represents a novel species that can be differentiated genotypically and phenotypically from its nearest phylogenetic neighbours, and for which we propose the name Rhizobium pusense sp. nov.

**Description of Rhizobium pusense sp. nov.**

_Rhizobium pusense_ (pu.sen’s.e. N.L. neut. adj. _pusense_ belonging to Pusa, India, the geographical origin of the type strain).

Cells are motile and rod-shaped. Gram-negative. Able to grow at 16–41 °C and at pH 5.0–11.0. Optimal growth occurs at 28–30 °C and at pH 7.5. Colonies on YM agar medium are round, mucoid, smooth and cream–white. Anaerobic growth occurs in the presence or absence of potassium nitrate. Catalase- and oxidase-positive. Utilizes adonitol, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, α-D-glucose, maltose, D-mannitol, D-mannose, methyl β-D-glucoside, D-sorbitol, sucrose, trehalose, turanose, L-asparagine, L-glutamic acid, L-proline, L-threonine and L-ornithine as carbon sources, but not α-cyclodextrin, pyruvic acid methyl ester, γ-hydroxybutyric acid, itaconic acid, malonic acid, sebacic acid, succinamic acid, glucuronamide, L-alaninamide, putrescine, 2-aminoethanol or DL-α-glycerol phosphate. The fatty acid profile comprises...
C18:1ω7c, C16:0, C19:0 cyclo ω8c iso-C16:1, C16:0 3-OH, C16:1ω7c and C18:0. The G+C content of the DNA of the type strain is 59.0 mol%.

The type strain, NRCBP10T (=LMG 25623T=ICM 16209T=NCIMB 14639T), was isolated from rhizosphere soil of chickepa (Cicer arietinum L.).

Acknowledgements

We thank Professor J. P. Euzéby (Ecole Nationale Vétérinaire, France) for etymological advice. We are grateful to Dr J. L. Swezy, ARS Culture and Patent Culture Collections, US Department of Agriculture, the BCCM/LMG Bacteria collection, Universiteit Gent, Belgium, and DSMZ, Germany, for providing other type strains used in this study. We thank Dr Aqbal Singh, NRC on Plant Biotechnology, Pusa, New Delhi, for his encouragement in the early stage of this work. We are also grateful to the All India Institute of Medical Sciences, New Delhi, for electron microscope facilities. This work was supported by the Department of Biotechnology, Ministry of Science and Technology, Government of India.

References


Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S RNA sequence analysis in the


