Rhizobium pusense sp. nov., isolated from the rhizosphere of chickpea (*Cicer arietinum* L.)

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A novel bacterial strain, designated NRCPB10^T, was isolated from rhizosphere soil of chickpea (*Cicer arietinum* L.) in Pusa, New Delhi, India. The 16S rRNA gene sequence of strain NRCPB10^T showed highest similarity (98.9%) to that of *Rhizobium radiobacter* NCPPB 2437^T, followed by *Rhizobium larrymoorei* AF3-10^T (97.7%) and *Rhizobium rubi* IFO 13261^T (97.4%).

Phylogenetic analysis of strain NRCPB10^T based on the housekeeping genes *recA* and *atpD* confirmed its position as distinct from recognized *Rhizobium* species. Levels of DNA–DNA relatedness between strain NRCPB10^T and *R. radiobacter* ICMP 5785^T, *R. larrymoorei* LMG 21410^T and *R. rubi* ICMP 6428^T were 51.0, 32.6 and 27.3%, respectively. Cellular fatty acids of strain NRCPB10^T were C_{18:1}ω7c (58.9%), C_{16:0} (15.5%), C_{19:0} cyclo ω8c (11.5%), iso-C_{16:1} (5.8%), C_{16:0} 3-OH (4.5%), C_{16:1}ω7c (2.1%) and C_{18:0} (1.3%). The G + C content of the genomic DNA of strain NRCPB10^T was 59.0 mol%. Strain NRCPB10^T did not nodulate chickpea plants or induce tumours in tobacco plants. Phenotypic and physiological properties along with SDS-PAGE of whole-cell soluble proteins differentiated strain NRCPB10^T from its closest phylogenetic neighbours. On the basis of data from the present polyphasic taxonomic study, strain NRCPB10^T 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 NRCPB10^T (=LMG 25623^T=JCM 16209^T=NCIMB 14639^T).

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-Chavez & Najera, 1989; Quigley *et al.*, 1997), rhizosphere and plant roots (Segovia *et al.*, 1991; Yanni *et al.*, 1997; Rosenblueth & Martínez-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 NRCPB10^T, isolated from the rhizosphere of a chickpea plant. Based on the results of our polyphasic taxonomic study, strain NRCPB10^T is considered to represent a novel species of the genus *Rhizobium*.

Strain NRCPB10^T 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

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA* and *atpD* gene sequences of strain NRCPB10^T are FJ969841, HQ166059 and HQ114263, respectively.

One supplementary table and two supplementary figures are available with the online version of this paper.

muroid cream–white colour was picked as being representative of several similar colonies, designated strain NRCPB10^T 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 NRCPB10^T 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 TM (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 NRCPB10^T 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 NRCPB10^T 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 NRCPB10^T 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 NRCPB10^T 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 NRCPB10^T and the type strains of related *Rhizobium* species

Strains: 1, NRCPB10^T; 2, *R. radiobacter* ICMP 5785^T; 3, *R. larrymoorei* LMG 21410^T; 4, *R. rubi* ICMP 6428^T. All tests were performed under the same conditions.

Characteristic	1	2	3	4
Growth with 4 % NaCl	+	–	–	–
Carbon-source utilization				
Dextrin, inosine	+	–	–	+
Glycogen, L-arabinose, lactulose, DL-lactic acid	–	+	+	–
Tween 40, Tween 80, <i>cis</i> -aconitic acid, D-galacturonic acid, β-hydroxybutyric acid, propionic acid	–	+	–	+
N-Acetyl-D-galactosamine, N-acetyl-D-glucosamine, α-hydroxybutyric acid, α-ketobutyric acid	–	+	+	+
α-Lactose, L-pyrogutamic acid	+	+	–	–
Succinic acid monomethyl ester, D-glucosaminic acid, citric acid, <i>p</i> -hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, L-leucine, γ-aminobutyric acid, 2,3-butanediol, phenylethylamine, thymidine, DL-carnitine, urocanic acid, i-erythritol, L-phenylalanine	–	–	–	+
Acetic acid, D-galactonic acid lactone, D-gluconic acid, D-alanine, glycyl L-aspartic acid, hydroxy-L-proline	+	+	–	+
D-Glucuronic acid, raffinose, formic acid	–	+	–	–
Succinic acid, bromosuccinic acid	–	–	+	+
Glycyl L-glutamic acid	+	–	–	–
D-Serine, uridine, L-histidine, L-alanyl glycine, quinic acid, D-saccharic acid, L-rhamnose	–	–	+	–
L-Serine, L-aspartic acid	+	–	+	+
α-D-Glucose 1-phosphate, D-glucose 6-phosphate, glycerol, L-alanine, <i>myo</i> -inositol, gentiobiose, melibiose, D-psicose, xylitol	+	+	+	–

The DNA G+C content of strain NRCPB10^T 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 NRCPB10^T and its close relatives *Rhizobium radiobacter* ICMP 5785^T, *Rhizobium larrymoorei* LMG 21410^T and *Rhizobium rubi* ICMP 6428^T 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 NRCPB10^T comprised C_{18:1}ω7c (58.9 %), C_{16:0} (15.5 %), C_{19:0} cyclo ω8c (11.5 %), iso-C_{16:1} (5.8 %), C_{16:0} 3-OH (4.5 %), C_{16:1}ω7c (2.1 %) and C_{18:0} (1.3 %). This profile of fatty acids matched those of the reference strains. However, strain NRCPB10^T could be differentiated based on the absence of C_{13:1} and C_{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 algorithm, respectively. Tree topologies and statistical significance of branch points were tested based on 1000 bootstrap resamplings of the data (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 NRCPB10^T and compared with those of closely related strains retrieved from GenBank. Strain NRCPB10^T showed 98.9 % 16S rRNA gene sequence similarity to *R. radiobacter* NCPPB 2437^T, 97.7 % to *R. larrymoorei* AF3-10^T and 97.4 % to *R. rubi* IFO 13261^T. In the neighbour-joining tree based on 16S rRNA gene sequences, strain NRCPB10^T 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 NRCPB10^T were

undertaken according to Gaunt *et al.* (2001). Levels of *recA* and *atpD* gene sequence similarity between strain NRCPB10^T 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 NRCPB10^T 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 NRCPB10^T, *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 rRNA 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 NRCPB10^T, *R. radiobacter* ICMP 5785^T, *R. larrymoorei* LMG 21410^T and *R. rubi* ICMP 6428^T. 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-³²P]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 NRCPB10^T, mean DNA–DNA reassociation values were 51 % with *R. radiobacter* ICMP 5785^T, 32 % with *R. larrymoorei* LMG 21410^T and 27.3 % with *R. rubi* ICMP 6428^T (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 NRCPB10^T 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 10 000 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)

Fig. 1. Neighbour-joining tree showing the phylogenetic relationship between strain NRCPB10^T 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.

Table 2. 16S rRNA, *recA* and *atpD* gene sequence similarity and DNA–DNA relatedness among strain NRCPB10^T and the type strains of closely related *Rhizobium* speciesValues are shown relative to strain NRCPB10^T.

Strain	Gene sequence similarity (%)			DNA–DNA relatedness (%)
	16S rRNA	<i>recA</i>	<i>atpD</i>	
NRCPB10 ^T	100.0	100.0	100.0	100.3 ± 3.5
<i>R. radiobacter</i> ICMP 5785 ^T	98.9	93.8	94.3	51.0 ± 1.4
<i>R. larrymoorei</i> LMG 21410 ^T	97.7	88.8	89.8	32.6 ± 2.3
<i>R. rubi</i> ICMP 6428 ^T	97.4	86.8	89.6	27.3 ± 5.2

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 NRCPB10^T were different from those of its closest related

reference strains (Supplementary Fig. S2). Based on this dendrogram, the level of protein similarity between strain NRCPB10^T and *R. radiobacter* ICMP 5785^T was 93 %.

Symbiotic properties are important features of rhizobia, and hence the presence of symbiotic genes in strain NRCPB10^T was examined by attempting to amplify the *nodA* (Haukka *et al.*, 1998) and *nifH* (Eardly *et al.*, 1992;

Fig. 2. Neighbour-joining phylogenetic tree showing the relationship between strain NRCPB10^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.

Fig. 3. Neighbour-joining phylogenetic tree showing the relationship between strain NRCPB10^T and the type strains of related species, based on *atpD* gene sequences. Bootstrap values of 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 NRCPB10^T.

To test the nodulation ability of strain NRCPB10^T, 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 10⁸ 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 µE m⁻² s⁻¹. The day temperature was 23 °C, the night temperature was 15 °C and the relative humidity was 60 %. Wild-type NRCPB10^T was also inoculated in tobacco plants (*Nicotiana tabacum*) to check for the formation of tumours. Strain NRCPB10^T 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 NRCPB10^T

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'se. 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

C_{18:1}ω7c, C_{16:0}, C_{19:0} cyclo ω8c, iso-C_{16:1}, C_{16:0} 3-OH, C_{16:1}ω7c and C_{18:0}. The G + C content of the DNA of the type strain is 59.0 mol%.

The type strain, NRCPB10^T (=LMG 25623^T=JCM 16209^T=NCIMB 14639^T), was isolated from rhizosphere soil of chickpea (*Cicer arietinum* L.).

Acknowledgements

We thank Professor J. P. Euzéby (Ecole Nationale Vétérinaire, France) for etymological advice. We are grateful to Dr J. Young, Landcare Research New Zealand Limited, Auckland, New Zealand, for providing *R. radiobacter* ICMP 5785^T and *R. rubi* ICMP 6428^T used in this study. We are also grateful to Dr J. L. Swezey, 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.

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