***Rhizobium yanglingense* sp. nov., isolated from arid and semi-arid regions in China**

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A novel rhizobial group, cluster 9, defined in previous research [Tan, Z. Y., Wang, E. T., Peng, G. X., Zhu, M. E., Martínez-Romero, E. & Chen, W. X. (1999). *Int J Syst Bacteriol* 49, 1457–1469], was further characterized by determination of DNA base composition, whole-cell protein SDS-PAGE analysis, DNA–DNA hybridization, 16S rRNA gene sequencing and host specificity. These isolates were collected from the wild legumes *Amphicarpaea trisperma*, *Coronilla varia* and *Gueldenstaedtia multiflora* growing in arid and semi-arid regions in north-western China. Isolates within cluster 9 grouped into a single cluster above a similarity level of 90.6% in a cluster analysis based on protein SDS-PAGE, and they were differentiated from defined rhizobial species. Comparative analysis of 16S rRNA gene sequences showed that isolate CCBAU 71623^T, representing cluster 9, was most related to *Rhizobium gallicum* and *Rhizobium mongolense*. The DNA–DNA homologies were lower than 42.4% among cluster 9 and defined species, including *R. gallicum* and *R. mongolense*. These data indicated that cluster 9 was a unique genomic species. Isolates within this cluster could share their host plants. They could not nodulate *Galega orientalis* and *Leucaena leucocephala* and formed ineffective nodules on *Phaseolus vulgaris*. This group could also be differentiated from defined species by phenotypic characteristics. It is therefore proposed as a new species, *Rhizobium yanglingense*, with isolate CCBAU 71623 as the type strain.

Keywords: *Rhizobium yanglingense*, *Amphicarpaea trisperma*, *Coronilla varia*, *Gueldenstaedtia multiflora*, phylogeny

INTRODUCTION

The description of new taxa of root- and stem-nodule bacteria has been accelerated by using a polyphasic approach (Vandamme *et al.*, 1996) to characterize new isolates from various leguminous plants. Among the 36 defined species within the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, seven species, including *Rhizobium mongolense* (van Berkum *et al.*, 1998), *Rhizobium huautlense* (Wang *et al.*, 1998), *Mesorhizobium plurifarium* (de Lajudie *et al.*, 1998b), *Mesorhizobium amorphae* (Wang *et al.*, 1999a), *Rhizobium undicola* (de Lajudie *et*

al., 1998a), *Sinorhizobium arboris* and *Sinorhizobium kostense* (Nick *et al.*, 1999), have been described in the last 3 years. The characterization of new isolates and the subsequent description of new taxa have improved our knowledge about the diversity of rhizobia and have offered a better view of the phylogenetic relationships and the evolution of these bacteria. Reviews of the taxonomy and phylogeny of rhizobia have been published by van Berkum & Eardly (1998) and Wang & Martínez-Romero (2000).

In addition, new data on rhizobial diversity are accumulating rapidly; for example, the diverse rhizobial populations associated with *Leucaena leucocephala* have been classified into 12 rDNA types, including *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* (Wang *et al.*, 1999b). Some novel lineages have been revealed by characterization of various *Bradyrhizobium* populations associated with different

Abbreviation: UPGMA, unweighted pair group method with arithmetic averages.

The GenBank accession numbers for the 16S rRNA sequences of strains CCBAU 71462 and CCBAU 71623^T are AF195031 and AF003375, respectively.

Table 1. Strains and isolates used in this research

Strain*	Name of host	Origin†
Cluster 9 (<i>R. yanglingense</i>)		
CCBAU 71462 (SH 2462)	<i>Coronilla varia</i>	Gansu
CCBAU 71012 (SH 246012)	<i>Coronilla varia</i>	Gansu
CCBAU 71113 (SH 17113)	<i>Coronilla varia</i>	Shaanxi
CCBAU 71718 (SH 1718)	<i>Coronilla varia</i>	Shaanxi
CCBAU 71623 ^T (SH 22623 ^T)	<i>Gueldenstaedtia multiflora</i>	Gansu
CCBAU 71465 (SH 1456)	<i>Amphicarpaea trisperma</i>	Shaanxi
CCBAU 71121 (SH 27121)	<i>Amphicarpaea trisperma</i>	Gansu
CCBAU 71931 (SH 28931)	<i>Amphicarpaea trisperma</i>	Gansu
CCBAU 71975 (SH 0975)	<i>Amphicarpaea trisperma</i>	Gansu
New isolates		
CCBAU 71414 (SH 414)	<i>Coronilla varia</i>	Shaanxi
CCBAU 71423 (SH 423)	<i>Gueldenstaedtia multiflora</i>	Shaanxi
CCBAU 71445 (SH 445)	<i>Coronilla varia</i>	Shaanxi
Reference strains		
<i>R. leguminosarum</i> USDA 2370 ^T	bv. <i>viceae</i>	United States
<i>R. tropici</i> CIAT 899 ^T	<i>Phaseolus vulgaris</i>	Columbia
<i>R. tropici</i> A CFN 299	<i>Phaseolus vulgaris</i>	Brazil
<i>R. mongolense</i> USDA 1844 ^T	<i>Meliloti luthenica</i>	Inner Mongolia
<i>R. galegae</i> HAMBI 540 ^T	<i>Galega orientalis</i>	Finland
<i>R. hainanense</i> I66 ^T	<i>Desmodium smuatum</i>	Hainan
<i>R. gallicum</i> USDA 2918 ^T	<i>Phaseolus vulgaris</i>	France
<i>R. gallicum</i> FL27	<i>Phaseolus vulgaris</i>	Mexico
<i>R. giardinii</i> H152 ^T	<i>Phaseolus vulgaris</i>	France

* CCBAU, Culture Collection of Beijing Agricultural University, China; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, Beltsville, MD, USA; CIAT, *Rhizobium* Collection, Centro Internacional de Agricultura Tropical, Cali, Columbia; CFN, Centro de Investigación sobre Fijación de nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, México; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland.

† Gansu, Shaanxi, Inner Mongolia and Hainan, provinces of China.

hosts (Barrera *et al.*, 1997; Dupuy *et al.*, 1994; Lafay & Burdon, 1998; van Rossum *et al.*, 1995). All these data indicate the existence of unknown rhizobia in nature and new taxa are expected when further investigation is performed on novel isolates or groups.

In our previous study, novel groups, clusters 9, 14 and 15, were found in rhizobial isolates from the wild legumes *Caragana pruinosa*, *Caragana microphylla*, *Caragana intermedia*, *Amorpha fruticosa*, *Sophora viciifolia*, *Coronilla varia*, *Amphicarpaea trisperma*, *Glycyrrhiza uralensis* and *Gueldenstaedtia multiflora* growing in the arid and semi-arid regions in the north-western areas of China. They were identified as *Rhizobium* and *Mesorhizobium* by their phenotypic features and by comparative RFLP analysis of PCR-amplified 16S rRNA genes (Tan *et al.*, 1999). In the present research, new isolates belonging to cluster 9 were obtained from host plants *Coronilla varia* and *Gueldenstaedtia multiflora*. These new isolates, together with those used in the previous study (Tan *et al.*, 1999), were further characterized. The object was to clarify the taxonomic position of cluster 9.

METHODS

Strains and isolates. All strains and isolates used in this research are listed in Table 1. Three new isolates were obtained from the root nodules of *Coronilla varia*, *Amphicarpaea trisperma* and *Gueldenstaedtia multiflora* growing in the fields of arid and semi-arid regions where the cluster 9 isolates were found (Tan *et al.*, 1999). All bacteria were subcultured in YMA medium (Vincent, 1970) and maintained in 20% glycerol solution at -20°C .

SDS-PAGE of whole-cell proteins. The medium and procedures described previously (Reinhold-Hurek *et al.*, 1993a, b) were used for growing the bacteria and for harvesting the cells. Fresh cells were weighed and suspended in extraction buffer (Laemmli, 1970) at a concentration of 150 mg ml^{-1} . Procedures for extraction of whole-cell proteins and electrophoresis described by Kiredjan *et al.* (1986) and Schagger & von Jagow (1987) were used. Two independent samples were prepared from strains CCBAU 71623^T and CCBAU 71462 for checking reproducibility. Universal Software 1D Advanced from AABI was used for normalization of the protein profiles and numerical analysis using the unweighted pair group method with arithmetic averages (UPGMA; Sneath & Sokal, 1973). A simple band match method was used for clustering. Bands with migration

distances less than 3.0 % different were defined as the same. The correlation coefficient is expressed as percentage similarity.

DNA base composition and DNA–DNA hybridization. DNAs were isolated and purified by the method described by Marmur (1961). DNA base composition was determined spectrophotometrically as described by De Ley (1970). DNA–DNA relatedness was determined by the initial renaturation rate method (De Ley *et al.*, 1970) in $2 \times$ SSC at 68 °C.

16S rRNA gene sequencing. Universal primers 25f and 1492r (Lane, 1991) and the procedures of Hurek *et al.* (1997) were used for PCR amplification of the complete 16S rRNA gene from strain CCBAU 71462. A single strand of this PCR product was sequenced directly using the primers 35fC, 342fC, 530mfC and 930fC and the procedures described by Hurek *et al.* (1997). Double-strand sequencing was performed for a cloned fragment of the PCR-amplified 16S rRNA gene from strain CCBAU 71623^T as described previously (Chen *et al.*, 1997; Tan *et al.*, 1997). Alignment of the sequences obtained and related sequences from the GenBank database were performed using the RDP program (Maidak *et al.*, 1999). The method of Jukes & Cantor (1969) was used to calculate the distances among the aligned sequences. Tree topology was inferred by the neighbour-joining method (Saitou & Nei, 1987), and the phylogenetic tree was constructed using the TREECON package (van de Peer & De Wachter, 1994). This package was also used to generate bootstrap confidence values (Felsenstein, 1985) from 100 replications of each sequence.

Cross-nodulation tests. Seeds of *Gueldenstaedtia multiflora* and *Coronilla varia* were scarified and seeds of *Amphicarpaea trisperma* were treated for 5 min with concentrated H₂SO₄. Standard procedures (Vincent, 1970) for surface sterilization, germination and inoculation of the seeds were used. The growth conditions of inoculated plants were described previously (Chen *et al.*, 1995).

RESULTS

SDS-PAGE of whole-cell proteins

Three new isolates (CCBAU 71445, CCBAU 71414 and CCBAU 71423) and reference strains for cluster 9 and defined species were compared by SDS-PAGE of proteins. The three new isolates and four cluster 9 strains had similar electrophoretic patterns and they formed a group at a similarity level of 90 % in a cluster analysis based upon the protein patterns (Fig. 1). The protein patterns of reference strains for related species were different from each other and from the cluster 9 strains. Different rhizobial species or groups were separated well below the similarity level of 90 %, with the exception of *R. gallicum* FL27 and *R. giardinii*, which had a similarity of 91 % (Fig. 1).

DNA base composition and DNA–DNA hybridization

The G+C content of new isolates CCBAU 71423, CCBAU 71414 and CCBAU 71445 was 60, 62 and 63 mol %, respectively, within the range of cluster 9 (59–63 mol %) (Tan *et al.*, 1999). The DNA relatedness among the reference strain CCBAU 71623^T and the new isolates CCBAU 71423, CCBAU 71414 and

Fig. 1. Dendrogram showing the relationships of *Rhizobium* species derived from SDS-PAGE protein patterns by UPGMA clustering using simple band match. The numbers show the similarity levels of the band match.

CCBAU 71445 was 93.5, 90.5 and 86.1 %, respectively, similar to those among other cluster 9 strains (72.3–100 %) (Tan *et al.*, 1999). The DNA relatedness among *R. gallicum* type strain USDA 2918^T and the cluster 9 strains CCBAU 71623^T, CCBAU 71718, CCBAU 71113 and CCBAU 71012 was 37.9 (Tan *et al.*, 1999), 13.9, 42.4 and 29.5 %, respectively, similar to those among strain CCBAU 71623^T and type strains of related species (< 31 %) (Tan *et al.*, 1999).

Sequence analysis of 16S rRNA gene

Isolates CCBAU 71462 and CCBAU 71623^T shared 99.2 % 16S rRNA gene sequence similarity. Only the sequence of CCBAU 71623^T was used for reconstruction of the phylogenetic tree (Fig. 2). In this tree, cluster 9 strains were grouped into the genus *Rhizobium* and most related to the sub-branch containing *R. gallicum* and *R. mongolense*. The sequence similarities were 99.2 % between cluster 9 and *R. gallicum*, and 99.0 % between cluster 9 and *R. mongolense*. Similarity levels among cluster 9 and defined *Rhizobium* species were 96.0–99.2 %. Cluster 9 strains and other root- or stem-nodule bacteria shared 89.2–97.0 % 16S rRNA gene sequence similarity.

Cross-nodulation

Cross-nodulation of strains or isolates within cluster 9 was checked. Isolates from *Amphicarpaea trisperma*, *Coronilla varia* and *Gueldenstaedtia multiflora* could share their host plants. None of the isolates within

Fig. 2. Phylogenetic tree constructed from a comparative analysis of 16S rRNA gene sequences showing the relationships among *R. yanglingense* and other species within the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. The GenBank accession numbers of the sequences are given in parentheses. Sequences were aligned using the RDP (Ribosomal Database Project) program (Maidak *et al.*, 1999). The Jukes–Cantor distance (Jukes & Cantor, 1969), bootstrap (Felsenstein, 1985) and neighbour-joining method (Saitou & Nei, 1987) were used to construct this phylogenetic tree using the TREECON package (van de Peer & De Wachter, 1994). Bootstrap values greater than 50% are indicated at the branch points. The scale bar shows 3% nucleotide substitutions.

cluster 9 could nodulate *Galega orientalis* or *Leucaena leucocephala*. Strain CCBau 71623^T also nodulated *Glycyrrhiza pallidiflora* and *Phaseolus vulgaris*, but the nodules on *Phaseolus vulgaris* formed by this strain were inefficient and decayed very soon since most of the mature nodules were white or green.

DISCUSSION

In a previous numerical taxonomic study, nine rhizobial isolates from *Amphicarpea trisperma*, *Coronilla varia* and *Gueldenstaedtia multiflora* formed a unique cluster, cluster 9. Three representative isolates of this cluster had identical PCR-based 16S rRNA gene RFLP patterns, which were closely related to those of *R. mongolense* and of *Rhizobium gallicum*. These close relationships were also confirmed by the high similarities of partial 16S rRNA gene sequences. DNA homologies greater than 70% were obtained among the cluster 9 strains, but the relationships within this cluster and with the defined species were not completely described in the previous work (Tan *et al.*, 1999).

In the present research, three new isolates were included and were identified as members of cluster 9 by analysis based on protein SDS-PAGE (Fig. 1) and DNA–DNA hybridization. The results of protein SDS-PAGE (Fig. 1), DNA relatedness and numerical taxonomy (Tan *et al.*, 1999) indicated that the fast-

growing, acid-producing rhizobial isolates from *Amphicarpea trisperma*, *Coronilla varia* and *Gueldenstaedtia multiflora* were a single group different from all the defined species. Comparative analysis of 16S rRNA sequences (Fig. 2) showed that this group was a member of the genus *Rhizobium*, and that it was closely related to *R. gallicum* and *R. mongolense* (> 99% 16S rRNA gene sequence similarity). However, the DNA–DNA homologies among the isolates of cluster 9 and type strains of *R. gallicum* and *R. mongolense* were lower than 42.4%.

In addition, distinctive features for cluster 9 were described in our previous report (Tan *et al.*, 1999) and other publications (Amarger *et al.*, 1997; van Berkum *et al.*, 1998; Wang *et al.*, 1998). PCR-based RFLP of 16S rRNA genes could be a useful molecular tool to differentiate cluster 9 from other defined species. Utilization of L-tryptophan as sole nitrogen source, and resistance to 5 µg streptomycin ml⁻¹ and to 1.0% (w/v) NaCl could differentiate cluster 9 from *R. gallicum*, one of the most related species. Resistance to 5 µg streptomycin ml⁻¹ and to 1.0% NaCl were also features which distinguished cluster 9 from *R. mongolense*. *R. gallicum* and *R. mongolense* also formed effective nodules on common bean plants, on which cluster 9 strains formed ineffective nodules.

Based upon the polyphasic taxonomy performed in this research and in the previous work (Tan *et al.*, 1999) and upon the current criteria to describe new

rhizobial taxa (Graham *et al.*, 1991), we propose cluster 9 as a new species, *Rhizobium yanglingense*.

Description of *Rhizobium yanglingense* sp. nov.

Rhizobium yanglingense (yang.ling.en'se N.L. adj. *Yangling* a town in Shianxi province of China, where the majority of strains within this species were isolated).

Aerobic, Gram-negative, non-spore-forming rods that are 0.5–1.0 µm wide by 2.0–3.5 µm long. Motile by means of a single polar flagellum. Colonies on YMA are semi-translucent, opalescent and usually 2.0–3.0 mm in diameter after 3 d incubation at 28 °C. Optimum temperature 25–30 °C, and can grow at 37 °C but not at 10 or 40 °C. Optimum pH 6–8 and can grow at pH 5–9, but not at pH 4 or 10. Generation time is 2.8–3.2 h in PY broth (Tan *et al.*, 1999), as determined by the spectrophotometric method. Strains produce a lot of polysaccharide and acid on YMA medium. They do not produce 3-ketolactose from lactose. Most strains do not grow in LB medium (Yanagi & Yamasato, 1993), with the exception of CCBAU 71121 and CCBAU 7131. No strains grow in media containing formate, salicylate, D-glutamic acid, L-methionine, β-alanine or L-threonine as sole carbon source. All of them can use L-alanine, L-glutamic acid or L-proline as sole nitrogen source. All strains are sensitive to 300 µg ml⁻¹ of doxycycline, gentamicin and neomycin, and to 0.1 % (w/v) bromothymol blue and gentian violet, but are resistant to 5–100 µg bacitracin ml⁻¹ and 5–50 µg erythromycin ml⁻¹. Growth is inhibited by 5.0 % (w/v) NaCl on YMA. Representative isolates from different hosts in cluster 9 show different *Eco*RI or *Bam*HI restriction patterns of *nodDAB* with 2–4 bands and a single *nifH* band (Tan *et al.*, 1999). The G + C content of the DNA is 59–63 mol % (*T_m*). The type strain is CCBAU 71623^T (SH 22623^T). Its DNA G + C content is 62 mol % (*T_m*). The strains have been deposited in the Cultural Collection of Beijing Agricultural University (CCBAU), China, and in the Culture Collection of Laboratorium voor Microbiologie, Universiteit Gent (LMG), Belgium.

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