Rhizobium indigoferae* sp. nov. and *Sinorhizobium kummerowiae* sp. nov., respectively isolated from *Indigofera* spp. and *Kummerowia stipulacea

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Forty-eight rhizobial isolates from root nodules of *Indigofera* and *Kummerowia*, two genera of annual or perennial wild legumes growing in the Loess Plateau in north-western China, were characterized by a polyphasic approach. Two main groups, cluster 1 and cluster 2, were defined based upon the results of numerical taxonomy, SDS-PAGE of whole-cell proteins and DNA relatedness. All the isolates within cluster 1 were isolated from *Indigofera* and they were identified as *Rhizobium* strains by 16S rRNA gene analysis. DNA relatedness of 29.5–48.9% was obtained among the cluster 1 isolates and the reference strains for defined *Rhizobium* species. Cluster 2 consisted of isolates from *Kummerowia stipulacea* and was identified as belonging to *Sinorhizobium* by 16S rRNA gene analyses. DNA relatedness varied from 5.2 to 41.7% among the isolates of cluster 2 and reference strains for *Sinorhizobium* species. Considering the existence of distinctive features among these two groups and related species within the genera *Rhizobium* and *Sinorhizobium*, we propose two novel species, *Rhizobium indigoferae* sp. nov. for cluster 1, with isolate CCBAU 71714^T (= AS 1.3046^T) as the type strain, and *Sinorhizobium kummerowiae* sp. nov. for cluster 2, with isolate CCBAU 71042^T (= AS 1.3045^T) as the type strain.

Keywords: *Rhizobium indigoferae*, *Sinorhizobium kummerowiae*, diversity, phylogeny

INTRODUCTION

Rhizobia are useful bacterial resources because of their symbiotic nitrogen-fixing ability. They form nodules on roots, and occasionally on stems, of many leguminous plants and reduce N₂ to ammonia, which can be used by the host plants. In the last two decades, the classification of rhizobia has developed rapidly, mostly by the application of polyphasic taxonomy and by the

continual investigation of rhizobial resources. At the time of writing, 31 species have been described within six genera: *Allorhizobium* (de Lajudie *et al.*, 1998b), *Azorhizobium* (Dreyfus *et al.*, 1988), *Bradyrhizobium* (Jordan, 1984; Kuykendall *et al.*, 1992; Xu *et al.*, 1995), *Mesorhizobium* (Jarvis *et al.*, 1982, 1997; Chen *et al.*, 1991, 1997; Nour *et al.*, 1994, 1995; de Lajudie *et al.*, 1998b; Wang *et al.*, 1999), *Rhizobium* (Amarger *et al.*, 1997; van Berkum *et al.*, 1998; Chen *et al.*, 1997; Jordan, 1984; Lindström, 1989; Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1993; Wang *et al.*, 1998) and *Sinorhizobium* (Chen *et al.*, 1988; de Lajudie *et al.*, 1994; Rome *et al.*, 1996). *Azorhizobium* and *Bradyrhizobium* are phylogenetically divergent from the other rhizobia. The remaining genera, *Allorhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*, are closely related to each other and they are intermingled with plant-pathogenic bacteria belonging to the genus

Published online ahead of print on 12 July 2002 as DOI 10.1099/ijs.0.02030-0.

Full details of the G+C contents and DNA–DNA relatedness of the individual novel isolates are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of strains CCBAU 71042^T and CCBAU 71714^T are AF364068 and AF364067.

Agrobacterium and non-symbiotic bacteria within the genus *Ensifer* (Casida, 1982). A novel group of root-nodule bacteria was recently identified as belonging to *Methylobacterium* (Sy *et al.*, 2001). Although most of the defined root-nodule bacteria are members of the α -subclass of the *Proteobacteria*, novel taxa of root-nodule bacteria, *Ralstonia taiwanensis* (Chen *et al.*, 2001) and *Burkholderia* sp. (Moulin *et al.*, 2001), have been reported, both being members of the β -*Proteobacteria*.

Legumes in the genera *Indigofera* and *Kummerowia* are annual or perennial wild plants. Most of them are drought-tolerant and they are held in high esteem as foliage, green manure crops or in preventing soil erosion (Allen & Allen, 1981). *Kummerowia* species are used as herbal medicine in China for treating diarrhoea and fever. These plants form root nodules but their microsymbionts have not been characterized systematically. The north-western Loess Plateau region of China, including the provinces of Shaanxi, Gansu and the Autonomous Region of Ningxia, is located in a temperate zone with a semi-arid climate and soils poor in organic matter. During the summer of 1992, we obtained some rhizobial isolates from different plants and different geographical areas in a survey of rhizobial resources in these regions. Previously, we have characterized isolates from *Amorpha fruticosa*, *Glycyrrhiza*, *Gueldenstaedtia* and other genera according to their geographical or host origin (Wang *et al.*, 1999; Tan *et al.*, 1999). Considering the potential value of *Indigofera* and *Kummerowia* in agriculture and reforestation and the unknown taxonomic position of the rhizobia associated with them, we decided to characterize isolates from *Indigofera* and *Kummerowia* plants in these regions with a polyphasic approach. The aims of the research were to check the diversity and to classify the isolates by both phenotypic and genetic methods.

METHODS

Isolates and strains. The isolates and reference strains used are listed in Table 1. The 21 isolates from *Indigofera* spp. and 27 isolates from *Kummerowia stipulacea* were fast-growing rhizobia that formed single colonies with diameters of 2–3 mm within 3 days on YMA. Rhizobia were isolated from fresh nodules collected from plants growing in the field by the standard method on YMA medium (Vincent, 1970). Single colonies were picked and checked for purity by repeated streaking and by microscopic examination. Nodulation with the original host plant of each isolate was checked in glass tubes, half-filled with vermiculite, as described by Vincent (1970). The temperature in the greenhouse was kept at 23 °C during the day and 12 °C during the night, with illumination of 10000–20000 lux for 14 h day⁻¹.

Phenotypic characterization and numerical taxonomy. A total of 136 phenotypic features, covering utilization of sole carbon and nitrogen sources, resistance to antibiotics, tolerance of dyes and chemicals, tolerance of different concentrations of NaCl, temperature and pH ranges for growth and some physiological and biochemical reactions were examined as described previously (Gao *et al.*, 1994) in

order to characterize the isolates and strains. The simple matching coefficient S_{sm} and UPGMA (Sneath & Sokal, 1973) were used for clustering analysis of phenotypic features. The mean similarity for each isolate within a cluster was estimated to present the phenotypic variation in the cluster, as described previously, and the isolate with the highest mean similarity was chosen as the central isolate or representative for the cluster (Sneath & Sokal, 1973). Generation time of the isolates was determined spectrophotometrically (Yelton *et al.*, 1983) in YM broth (Vincent, 1970).

SDS-PAGE of whole-cell proteins. Methods described previously (Tan *et al.*, 1997) were used for soluble protein extraction, SDS-PAGE and scanning of the protein patterns using an Extra-Scanner densitometer (LKB). Jaccard's coefficient (S_j) and UPGMA (Sneath & Sokal, 1973) were used for cluster analysis.

PCR-based RFLP of 16S rRNA genes. Procedures described previously (Wang *et al.*, 1998) were used for PCR amplification of complete 16S rRNA genes with the universal forward primer P1 and the universal reverse primer P6 (Tan *et al.*, 1997), for digestion of the PCR products with restriction endonucleases *MspI*, *HinfI*, *HaeI* and *RsaI* and for separation of the digested fragments in 3.0% agarose gels. Primer P1 (5'-CGgataccAGAGTTTGATCCTGGTC-AGAACGCT-3') corresponds to positions 8–37 and primer P6 (5'-CGgataccTACGGCTACCTTGTTACGACTTCA-CCCC-3') corresponds to positions 1479–1506 in the *Escherichia coli* 16S rDNA (Willems & Collins, 1993; Yanagi & Yamasato, 1993). Lower-case letters indicate the *Bam*HI restriction site.

Amplification, cloning and sequencing of 16S rDNA. This study was performed only for representative isolates of the novel clusters. Full-length 16S rRNA genes (1500 bp) were amplified from isolates by PCR in a 100 μ l reaction mixture using primers P1 and P6. Amplified 16S rDNA was purified from 0.8% low-melting-point agarose gel by the method of Wieslander (1979). Purified rDNA and plasmid vector pUC18 were restricted with *Bam*HI and ligated at 14 °C for 16 h. Ligated plasmids were transformed into *E. coli* DH5 α and transformants were selected using the blue/white screening procedure (Sambrook *et al.*, 1989). Plasmids containing 16S rDNA were extracted and purified according to the methods of Tiesman & Rizzino (1991). Purified plasmids were sequenced using an ABI377 automatic sequencer (Applied Biosystems). Two independent clones of PCR-amplified 16S rRNA genes from each isolate were sequenced.

Sequence analysis and reconstruction of the phylogenetic tree. The 16S rRNA gene sequences obtained and those of related bacteria obtained from GenBank were aligned using the PILEUP program in the Wisconsin package (Genetics Computer Group, 1995). Sequence similarities and a phylogenetic tree were generated and bootstrapped with 1000 subsamples using CLUSTAL W version 1.7 (Thompson *et al.*, 1994). The tree was visualized by using the program TreeView (Page, 1996).

Estimation of DNA G + C content and DNA–DNA relatedness. DNAs were extracted and purified by the method of Marmur (1961). DNA base composition was determined using the thermal melting protocol (De Ley, 1970) and using *E. coli* K-12 as standard. Levels of DNA relatedness were determined by the initial renaturation rate method (De Ley *et al.*, 1970).

Cross-nodulation tests. Standard methods were used (Vin-

Table 1. Isolates and reference strains used in this research

Culture collections/strain sources: CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China; CFN, Centro de Investigación sobre Fijación de Nitrógeno; CIAT, Centro Internacional de Agricultura Tropical, Cali, Colombia; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; LMG, Bacteria Collection of the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; NZP, Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; USDA, *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, USDA, Beltsville, MD, USA.

Strain/isolate	Host plant	Geographical origin	Year of isolation or reference
Cluster 1 (<i>Rhizobium indigoferae</i> sp. nov.)			
CCBAU 71042 ^T , CCBAU 71364, CCBAU 71260	<i>Indigofera amblyanthra</i>	Shaanxi, China	1996
CCBAU 71385, CCBAU 71845	<i>I. amblyanthra</i>	Gansu, China	1996
CCBAU 71849	<i>Indigofera carlesii</i>	Shaanxi, China	1996
CCBAU 71064, CCBAU 71860	<i>I. carlesii</i>	Gansu, China	1996
CCBAU 71864, CCBAU 71863, CCBAU 71843	<i>Indigofera potaninii</i>	Shaanxi, China	1996
CCBAU 71841, CCBAU 71846, CCBAU 71862, CCBAU 71861	<i>I. amblyanthra</i>	Shaanxi, China	1997
CCBAU 71710, CCBAU 71718	<i>I. amblyanthra</i>	Ningxia, China	1997
CCBAU 71848	<i>I. potaninii</i>	Shaanxi, China	1997
CCBAU 71865, CCBAU 71840	<i>I. carlesii</i>	Gansu, China	1997
Cluster 2 (<i>Sinorhizobium kummerowiae</i> sp. nov.)			
CCBAU 71713, CCBAU 711, CCBAU 71327, CCBAU 71714 ^T , CCBAU 71715	<i>Kummerowia stipulacea</i>	Shaanxi, China	1996
CCBAU 71463, CCBAU 71481, CCBAU 71726, CCBAU 71723, CCBAU 71725, CCBAU 71741, CCBAU 71735, CCBAU 71321, CCBAU 71390, CCBAU 71381, CCBAU 71738, CCBAU 71734	<i>K. stipulacea</i>	Shaanxi, China	1997
CCBAU 71480, CCBAU 71391, CCBAU 71400, CCBAU 71727	<i>K. stipulacea</i>	Gansu, China	1996
CCBAU 71743, CCBAU 71747, CCBAU 71401	<i>K. stipulacea</i>	Ningxia, China	1997
Other rhizobial isolates			
CCBAU 71150, CCBAU 71712	<i>K. stipulacea</i>	Shaanxi, China	1996
CCBAU 71269	<i>K. stipulacea</i>	Gansu, China	1996
CCBAU 71369	<i>I. amblyanthra</i>	Shaanxi, China	1996
Reference strains			
<i>Sinorhizobium meliloti</i> USDA 1002 ^T , 102F28	<i>Medicago sativa</i>		Jordan (1984)
<i>Sinorhizobium medicae</i> USDA 1037 ^T	<i>Medicago truncatula</i>	France	Rome <i>et al.</i> (1996)
<i>Sinorhizobium fredii</i> USDA 205 ^T , USDA 194	<i>Glycine soja</i>	Henan, China	Scholla & Elkan (1984)
<i>Sinorhizobium xinjiangense</i> CCBAU 110 ^T , CCBAU 108	<i>G. soja</i>	Xinjiang, China	Chen <i>et al.</i> (1988)
<i>Sinorhizobium teranga</i> LMG 7834 ^T	<i>Acacia laeta</i>	Senegal	de Lajudie <i>et al.</i> (1994)
<i>Sinorhizobium teranga</i> LMG 6463	<i>Sesbania rostrata</i>	Senegal	de Lajudie <i>et al.</i> (1994)
<i>Sinorhizobium saheli</i> LMG 7837 ^T	<i>Sesbania pachycarpa</i>	Senegal	de Lajudie <i>et al.</i> (1994)
<i>Mesorhizobium tianshanense</i> A-IBS ^T , 6	<i>Glycyrrhiza uralensis</i>	Xinjiang, China	Chen <i>et al.</i> (1995)
<i>Mesorhizobium loti</i> NZP 2213 ^T	<i>Lotus corniculatus</i>	New Zealand	Jarvis <i>et al.</i> (1997)
<i>Mesorhizobium loti</i> NZP 2227	<i>Lotus</i> sp.	New Zealand	Jarvis <i>et al.</i> (1997)
<i>Mesorhizobium huakuii</i> CCBAU 2609 ^T	<i>Astragalus sinicus</i>	Nanjing, China	Chen <i>et al.</i> (1991)
<i>Mesorhizobium huakuii</i> A106	<i>A. sinicus</i>	Hubei, China	Chen <i>et al.</i> (1991)
<i>Mesorhizobium ciceri</i> USDA 3383 ^T	<i>Cicer arietinum</i>	Spain	Nour <i>et al.</i> (1994)
<i>Rhizobium leguminosarum</i> bv. <i>viceae</i> USDA 2370 ^T		USA	Jordan (1984)
<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i> 127K17		USA	Jordan (1984)
<i>Rhizobium tropici</i> (type B) CIAT 899 ^T	<i>Phaseolus vulgaris</i>	Colombia	Martínez-Romero <i>et al.</i> (1991)
<i>Rhizobium tropici</i> (type B) BR853	<i>Leucaena leucocephala</i>	Brazil	Martínez-Romero <i>et al.</i> (1991)
<i>Rhizobium tropici</i> (type A) CFN 299	<i>P. vulgaris</i>	Brazil	Martínez-Romero <i>et al.</i> (1991)
<i>Rhizobium etli</i> CFN 42 ^T	<i>P. vulgaris</i>	Mexico	Segovia <i>et al.</i> (1993)
<i>Rhizobium hainanense</i> I66 ^T , H14	<i>Desmodium sinuatum</i>	Hainan, China	Chen <i>et al.</i> (1996)
<i>Rhizobium gallicum</i> USDA 2918 ^T	<i>P. vulgaris</i>	France	Amarger <i>et al.</i> (1997)
<i>Rhizobium gallicum</i> FL27	<i>P. vulgaris</i>	France	Amarger <i>et al.</i> (1997)
<i>Rhizobium mongolense</i> USDA 1844 ^T	<i>Medicago ruthenica</i>	Inner Mongolia, China	van Berkum <i>et al.</i> (1998)
<i>Rhizobium galegae</i> HAMBI 540 ^T	<i>Galega orientalis</i>	Finland	Lindström <i>et al.</i> (1989)
<i>Rhizobium galegae</i> HAMBI 1185	<i>Galega</i> sp.	UK	Lindström <i>et al.</i> (1989)
<i>Rhizobium huautlense</i> S02 ^T	<i>Sesbania herbacea</i>	Mexico	Wang <i>et al.</i> (1999)
<i>Rhizobium giardinii</i> USDA 2914 ^T	<i>P. vulgaris</i>	France	Amarger <i>et al.</i> (1997)
<i>Bradyrhizobium elkanii</i> USDA 76 ^T	<i>Glycine max</i>	USA	Kuykendall <i>et al.</i> (1992)
<i>Bradyrhizobium japonicum</i> USDA 6 ^T	<i>G. max</i>	Japan	Jordan (1982)

cent, 1970). Plants were grown in pots filled with vermiculite moistened with N-free plant nutrient solution (Vincent, 1970). Nodulation and nitrogen fixation were observed after 1 month. Nodulation by representatives of the novel groups was tested on selected recommended legumes (Graham *et al.*, 1991), including *Astragalus sinicus*, *Medicago sativa*, *Galega orientalis*, *Lotus corniculatus*, *Vigna sinensis*, *Glycine max*, *Trifolium repens*, *Phaseolus vulgaris* and *Caragana korshinskii*.

RESULTS

Phenotypic characterization and numerical taxonomy

In the phenotypic characterization, none of the strains or isolates tested used adipate, dextrin, inulin, starch, sorbitol, syringic acid, D-arginine, L-methionine or L-threonine as a sole carbon source. All strains were sensitive to 50–300 µg kanamycin ml⁻¹ and 100–300 µg

Fig. 1. Phenogram of numerical taxonomy showing the grouping results of the tested strains and isolates. Coefficient S_{sm} and UPGMA (Sneath & Sokal, 1973) were used for the clustering analysis of 102 variable features. Clusters were defined at the similarity level of 85%.

neomycin ml^{-1} . No strain could grow in medium supplemented with gentian violet (0.1%), methylene blue (0.1%) or 4.0 or 5.0% NaCl. No strain grew at pH 4.0 or at 4 or 40 °C. No 3-ketolactose was produced. All strains were resistant to the following antibiotics ($\mu\text{g ml}^{-1}$): ampicillin (5), bacitracin (5–300), chloramphenicol (5), erythromycin (5 and 50), neomycin (5), polymyxin (5) and streptomycin (5). All strains grew in medium supplied with bismark brown (0.1%), congo red (0.1%), erythrosin (0.1%), neutral red (0.1%), sodium deoxycholate (0.1%), sodium nitrite (0.1%) or NaCl (1.0 and 2.0%) and at pH 9.0 and 10.0.

A total of 102 variable features were used for numerical taxonomy. Based on the results of cluster analysis, the isolates formed two clusters while the reference strains were grouped according to species (Fig. 1). Eight of the nine isolates from *Indigofera* spp. formed cluster 1 and

eight of the ten isolates from *K. stipulacea* were grouped into cluster 2 (Fig. 1). The other isolates were single branches. The distinctive phenotype features for cluster 1 and 2 are presented in Table 2. Some important aspects of the two clusters are described below.

Cluster 1 consisted of eight fast-growing isolates from three host species within the genus *Indigofera* (*Indigofera amblyantha*, *Indigofera carlesii* and *Indigofera potaninii*). The mean similarity for the isolates ranged from 83% in the case of CCBAU 71260 to 97% in the case of CCBAU 71042^T; the latter isolate was chosen as the central isolate for the cluster. Colonies were 2–3 mm in diameter after 2 days on YMA at 28 °C. All the isolates in this cluster produced acid in YMA.

Cluster 2 contained seven isolates from *K. stipulacea*. The mean similarities ranged from 82 to 96%. Isolate CCBAU 71714^T was the central isolate. They were fast-growing, acid-producing bacteria with generation times of 2.0–3.0 h in PY broth. No growth was observed in Luria–Bertani broth for these isolates.

SDS-PAGE of whole-cell proteins

In order to increase the number of strains in clusters 1 and 2 and to investigate the genetic relationships among the isolates and defined species, we performed SDS-PAGE analysis of whole-cell proteins. The grouping results from the protein patterns (Fig. 2) were generally correlated with those from the numerical taxonomy (Fig. 1). The similarities of the protein patterns ranged from 88 to 96.5% in cluster 1 and 82 to 97% in cluster 2. The similarities between different clusters or the type strains for defined species were lower than 79%. The number of isolates was increased to 20 in cluster 1 (from *Indigofera* spp.) and to 25 in cluster 2 (from *K. stipulacea*).

PCR-RFLP of 16S rRNA genes

Based on the grouping results in numerical taxonomy, isolates CCBAU 71042^T, CCBAU 71385 and CCBAU 71064, representing cluster 1, isolates CCBAU 71714^T, CCBAU 71327 and CCBAU 71713, representing cluster 2, and reference strains for defined species were chosen for PCR-RFLP of 16S rRNA genes. The representative isolates of clusters 1 and 2 gave highly similar patterns, but one or two bands were different within each cluster. Differences were observed between the patterns of isolates from the two clusters and the defined species (not shown). In a cluster analysis of the RFLP patterns, cluster 1 grouped with *Rhizobium* species and cluster 2 fell in the genus *Sinorhizobium* (not shown).

Sequencing and phylogeny of 16S rRNA genes

16S rRNA gene sequences were obtained from isolate CCBAU 71042^T, representing cluster 1, and isolate CCBAU 71714^T, representing cluster 2. Sequences

Table 2. Distinctive features of the novel clusters and related species defined in numerical taxonomy

Phenons are identified as: 1, *R. etli* ($n = 2$); 2, *R. leguminosarum* ($n = 2$); 3, *R. tropici* ($n = 2$); 4, *R. galegae* ($n = 2$); 5, *R. hainanense* ($n = 2$); 6, *S. fredii* ($n = 2$); 7, *S. meliloti* ($n = 2$); 8, *S. teranga* ($n = 1$); 9, *S. saheli* ($n = 1$); 10, cluster 1 ($n = 8$); 11, cluster 2 ($n = 7$). Numbers given in the table are percentages of strains testing positive; +, all strains positive; –, all strains negative.

Feature	1	2	3	4	5	6	7	8	9	10	11
Utilization as sole carbon source:											
D-Amygdalin	–	+	–	50	–	–	–	–	–	13	–
D-Arabinose	+	+	+	+	+	+	+	+	–	–	–
D(+)-Arabitol	+	–	50	–	+	–	+	–	–	75	70
Calcium malonate	–	+	–	+	–	–	–	–	+	–	–
Dulcitol	–	+	–	+	+	–	–	+	–	+	+
meso-Erythritol	–	–	+	50	+	–	+	+	–	25	86
D(+)-Galactose	+	50	50	+	+	+	+	+	–	+	29
Lactose	–	+	50	+	–	50	+	–	–	88	–
D-Mannose	+	+	+	+	+	+	+	+	–	+	–
Sodium citrate	+	+	+	+	+	+	50	+	+	+	–
Salicin	+	+	–	+	–	–	–	+	–	+	70
D-Ribose	+	+	+	–	+	–	+	+	–	25	–
Sodium D-gluconate	+	–	–	50	+	–	50	–	–	75	–
D-Sorbitol	+	+	+	+	+	50	+	+	+	75	–
Sucrose	+	–	–	+	+	50	–	+	–	+	30
Tartrate	+	50	50	50	–	+	50	–	+	–	–
Trehalose	+	+	+	+	+	+	+	+	+	88	–
Xylose	+	+	+	–	+	50	+	–	+	+	14
DL-Asparagine	+	+	+	50	+	+	+	+	+	+	+
Glycine	+	–	+	50	+	50	+	–	–	+	+
Utilization as sole nitrogen source:											
L-Cysteine	+	+	+	+	+	–	+	+	+	+	–
D-Glutamic acid	–	–	+	+	+	50	50	–	–	88	+
Glycine	+	–	+	–	+	+	+	–	+	–	86
D-Valine	–	–	–	+	+	–	–	–	–	–	–
Antibiotic resistance ($\mu\text{g ml}^{-1}$):											
Ampicillin (100)	+	+	+	+	+	–	+	+	+	13	+
Chloramphenicol (100)	–	–	+	–	+	–	–	+	+	–	+
Erythromycin (100)	–	–	–	+	+	–	+	–	–	13	+
Kanamycin (5)	+	+	+	+	+	+	+	+	+	–	14
Streptomycin (300)	+	–	+	–	–	+	50	–	+	–	14
Chemical tolerance (%):											
Acridine hydrochloride (0.1)	–	–	+	–	+	–	–	+	+	–	–
Methyl green (0.1)	+	+	–	+	+	+	+	+	+	–	–
NaCl (3.0)	–	+	+	+	50	+	50	–	+	13	–
Growth at pH 5.0	–	–	+	+	+	–	–	–	–	–	–
Urease activity	+	+	+	+	+	–	+	+	+	25	+
Litmus milk acid coagulation	–	–	–	–	–	–	–	+	–	–	–
Litmus milk alkali production	–	+	–	+	–	+	+	–	+	75	–
Litmus milk peptonization	+	+	+	+	+	–	–	–	–	+	29
Reduction of:											
Litmus milk	+	–	+	–	+	–	–	–	–	25	+
Methylene blue	+	–	+	50	+	–	+	–	+	–	70
Nile blue	–	–	+	+	+	–	–	–	–	–	14

from two clones of each isolate were identical. Isolate CCBAU 71042^T grouped with *Rhizobium* species, most similar to *Rhizobium gallicum* and *Rhizobium mongolense* (Fig. 3). The 16S rDNA sequence similarity

between isolate CCBAU 71042^T and *R. mongolense* USDA 1844^T, *R. gallicum* USDA 2918^T, *Rhizobium tropici* CIAT 899^T, *R. tropici* CFN 299, *Rhizobium leguminosarum* USDA 2370^T, *Rhizobium etli* CFN 42^T

Fig. 2. Dendrogram showing the grouping results of SDS-PAGE patterns of whole-cell proteins extracted from the bacterial strains. The electrophoretic patterns were scanned by densitometer by using the S_j coefficient and UPGMA (Sneath & Sokal, 1973) as described by Tan *et al.* (1997). Clusters and defined species were separated at the similarity level of 82 %.

and *Rhizobium hainanense* I66^T was respectively 97.9, 97.7, 95.5, 95.4, 96.2, 95.8 and 96.3 %. Isolate CCBAU 71714^T grouped within the *Sinorhizobium* phylogenetic branch, most related to *Sinorhizobium xinjiangense* and *Sinorhizobium fredii*. The 16S rDNA sequence similarity between isolate CCBAU 71714^T and *S. xinjiangense* CCBAU 110^T, *S. fredii* USDA 205^T, *Sinorhizobium medicae* USDA 1037^T, *Sinorhizobium meliloti* USDA 1002^T, *Sinorhizobium saheli* LMG 7837^T and *Sinorhizobium terangaee* LMG 7834^T was respectively 98.4, 98.5, 97.7, 97.8, 98.2 and 96.6 %. The phylogenetic relationships among the species of the genera *Agrobacterium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* in our reconstructed tree (Fig. 3) were similar to those in trees reported previously (Chen *et al.*, 1997; de Lajudie *et al.*, 1994, 1998a, b; Tan *et al.*, 1997; Wang *et al.*, 1998, 1999).

DNA G + C contents and DNA–DNA hybridization

These experiments were designed according to the results presented above. The results of DNA G + C content estimation and DNA–DNA hybridization are shown in Table 3. Full details of the G + C contents and DNA–DNA relatedness of the individual novel isolates are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>). The DNA G + C contents for the isolates from clusters 1 and 2 were respectively 59.0–62.0 mol % and 59.1–61.8 mol %, which is in the range for *Rhizobium* (Jordan, 1984) and *Sinorhizobium*. The DNA–DNA relatedness among the isolates of each cluster was higher than 80 %: within cluster 1 the range was 80.6–94.9 % (mean 88.2 %) and for cluster 2 the range was 83.7–94.0 % (mean 89.1 %). DNA–DNA relatedness between the representative isolate of cluster 1 (CCBAU 71042^T) and reference strains for *Rhizobium* species was 29.5–48.9 %. Low DNA relatedness (5.2–41.7 %) was obtained between isolate CCBAU 71714^T, representing cluster 2, and reference strains for *Sinorhizobium* species (Table 3).

Symbiotic performance

The isolates of clusters 1 and 2 were able to nodulate both of the plants from which they were isolated. Isolate CCBAU 71042^T, representing cluster 1, did not nodulate *Astragalus sinicus*, *Medicago sativa*, *Galega orientalis*, *Lotus corniculatus*, *Vigna sinensis*, *Glycine max*, *Trifolium repens*, *Phaseolus vulgaris* or *Caragana korshinskii*. Strain CCBAU 71714^T, representing cluster 2, nodulated *Medicago sativa* but not *Astragalus sinicus*, *Galega orientalis*, *Lotus corniculatus*, *Vigna sinensis*, *Glycine max*, *Trifolium repens*, *Phaseolus vulgaris* or *Caragana korshinskii*.

DISCUSSION

In this study, we characterized 21 nodule isolates from *Indigofera* and 27 nodule isolates from *Kummerowia* species from the north-western Loess Plateau of China by means of a polyphasic approach. Two main clusters, 1 and 2, were obtained among the isolates by numerical taxonomy based on phenotypic characterization (Fig. 1). The grouping results from SDS-PAGE of proteins (Fig. 2), analysis of DNA–DNA relatedness (Table 3) and PCR-based RFLP of 16S rRNA genes supported the results of numerical taxonomy and indicated that these two clusters were also two genomic groups or species. We concluded that cluster 1 was a member of *Rhizobium* and cluster 2 was a member of *Sinorhizobium* based upon the comparative analysis of 16S rRNA gene sequences (Fig. 3). By analysis of DNA–DNA relatedness (Table 3), these two groups were different from related species in the genera *Rhizobium* and *Sinorhizobium*.

It is interesting that 20 isolates from *Indigofera* spp. and 24 isolates from *K. stipulacea* formed two groups, but they could share their host plants under laboratory

Fig. 3. Phylogram of 16S rRNA gene sequences showing the phylogenetic relationships of the two novel groups obtained in this study and related species. Sequences were aligned using PILEUP. The phylogenetic tree was reconstructed and bootstrapped with 1000 subsamples using CLUSTAL W version 1.7 (Thompson et al., 1994). TreeView was used for visualizing the tree. Bootstrap values greater than 50% are indicated at the branching points. Bar, 5% base substitution.

conditions. This observation may indicate that the host plant prefers some rhizobia for nodulation in nature. A similar situation was also observed among the rhizobia that nodulate *Sesbania herbacea*. Seedlings of *S. herbacea* can nodulate with other rhizobia within the genera *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* when *Rhizobium huautlense*, the natural microsymbiont for this plant, is absent (Wang & Martínez-Romero, 2000). Based on these results, we think that the original host(s) should be an important feature for description of the species and that they are more valuable for classification than cross-nodulation results under laboratory conditions.

Based on all the results, we propose two novel species, *Rhizobium indigoferae* sp. nov. for the cluster 1 isolates and *Sinorhizobium kummerowiae* sp. nov. for the cluster 2 isolates, according to the current criteria for description of new rhizobial species (Graham et al., 1991).

Description of *Rhizobium indigoferae* sp. nov.

Rhizobium indigoferae (in.di.go'fe.rae. N.L. gen. n. *indigoferae* of *Indigofera*, a genus of leguminous plants, referring to the host from which the bacterium was isolated).

Cells are aerobic, Gram-negative, non-spore-forming rods that are 0.4–0.7 × 1.5–2.6 µm. Colonies are

2–3 mm in diameter after 2 days at 28 °C and produce acid in YMA. Generation time is 2.2–3.8 h in PY broth. Can grow on YMA in the presence of 2.0% NaCl. No growth at 4 or 40 °C. Optimum pH is 6.5–7.5; no growth at pH 5.0. Uses dulcitol, D(+)-galactose, D-mannose, sodium citrate, salicin, sucrose, xylose and DL-asparagine, but not D-arabinose, calcium malonate or tartrate as sole carbon sources. Uses L-cysteine, but not glycine or D-valine as a sole nitrogen source. Sensitive to 100 µg kanamycin and chloramphenicol ml⁻¹ and 300 µg streptomycin ml⁻¹. No strain can grow in medium with acridine hydrochloride (0.1%) or methyl green (0.1%). Methylene blue reduction and nile-blue reduction are negative. Strains have been isolated from *Indigofera* spp. The DNA G+C content is 59.0–62.0 mol% (*T_m*).

The type strain is strain CCBAU 71042^T (= AS 1.3054^T). Its generation time is 2.6 h and its DNA G+C content is 60.1 mol% (*T_m*).

Description of *Sinorhizobium kummerowiae* sp. nov.

Sinorhizobium kummerowiae (kum.me.ro'wi.ae. N.L. gen. n. *kummerowiae* of *Kummerowia*, a genus of leguminous plants, referring to the host from which the bacterium was isolated).

Cells are aerobic, Gram-negative, non-spore-forming

Table 3. DNA G + C contents and DNA–DNA relatedness among the novel isolates and defined species

DNA G + C contents were determined by the T_m method. ND, Not determined. Full details of the G + C contents and DNA–DNA relatedness of the individual novel isolates are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org/>).

Strain(s) or isolate(s)	DNA G + C content (mol %)	DNA–DNA hybridization with:	
		CCBAU 71042 ^T (cluster 1)	CCBAU 71714 ^T (cluster 2)
Cluster 1 (<i>R. indigoferae</i> sp. nov.)			
CCBAU 71042 ^T	60.1	100	ND
19 other isolates	59.0–62.0	80.6–94.9	ND
Cluster 2 (<i>S. kummerowiae</i> sp. nov.)			
CCBAU 71714 ^T	61.6	ND	100
23 other isolates	59.1–61.8	ND	83.7–96.8
<i>R. leguminosarum</i> USDA 2370 ^T , 127K17	ND	36.4, 30.4	ND
<i>R. tropici</i> type B CIAT 899 ^T	ND	38.1	ND
<i>R. tropici</i> type A CFN 299	ND	48.9	ND
<i>R. etli</i> CFN 42 ^T	ND	41.7	ND
<i>R. hainanense</i> I66 ^T , H14	ND	32.2, 40.3	ND
<i>R. gallicum</i> USDA 2918 ^T	ND	30.5	ND
<i>R. mongolense</i> USDA 1844 ^T	ND	29.5	ND
<i>R. yanglingense</i> CCBAU 71623 ^T	ND	40.1	ND
<i>S. arboris</i> HAMBI 1552 ^T	ND	ND	24.5
<i>S. kostiense</i> HAMBI 1489 ^T	ND	ND	30.2
<i>S. meliloti</i> USDA 1002 ^T , 102F28	ND	ND	15.6, 25.9
<i>S. medicae</i> USDA 1037 ^T	ND	ND	5.2
<i>S. fredii</i> USDA 205 ^T , USDA 194	ND	ND	41.7, 39.6
<i>S. xinjiangense</i> CCBAU 110 ^T , CCBAU 108	ND	ND	16.5, 10.2
<i>S. teranga</i> LMG 7834 ^T , LMG 6463	ND	ND	18.7, 20.4
<i>S. saheli</i> LMG 7837 ^T	ND	ND	37.3

rods that are 0.3–0.6 × 1.8–3.0 µm. Colonies are 2–3 mm in diameter after 1–2 days at 28 °C and produce acid in YMA. Generation time is 2.0–3.0 h in PY broth. Sensitive to 3.0% (w/v) NaCl in YMA. No growth at 4 or 40 °C. Optimum pH is 6.5–7.5; no growth at pH 5.0. Uses dulcitol, DL-asparagine and glycine, but not D-amygdalin, D-arabinose, calcium malonate, lactose, tartrate, D-ribose, sodium D-glucuronate, trehalose, D-mannose or sodium citrate as sole carbon sources. Uses D-glutamic acid but not L-cysteine or D-valine as a sole nitrogen source. Not sensitive to 100 µg ampicillin, chloramphenicol or erythromycin ml⁻¹. No strain can grow in medium with acridine hydrochloride (0.1%) or methyl green (0.1%). No growth in Luria–Bertani broth. Strains have been isolated from *Kummerowia stipulacea*. The DNA G + C content is 59.1–61.8 mol % (T_m).

The type strain is strain CCBAU 71714^T (= AS 1.3046^T). Its generation time is 2.2 h and its DNA G + C content is 61.6 mol % (T_m).

ACKNOWLEDGEMENTS

This research was part of project no. 39130010 supported by the National Science Foundation of China.

REFERENCES

- Allen, O. N. & Allen, E. K. (1981). *The Leguminosae: a Source Book of Characteristics, Uses, and Nodulation*. Madison, WI: University of Wisconsin Press.
- Amarger, N., Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* **47**, 996–1006.
- Casida, L. E., Jr (1982). *Ensifer adhaerens* gen. nov., sp. nov.: a bacterial predator of bacteria in soil. *Int J Syst Bacteriol* **32**, 339–345.
- Chen, W. X., Yan, G. H. & Li, J. L. (1988). Numerical taxonomic study of fast-growing soybean rhizobia and proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. *Int J Syst Bacteriol* **38**, 392–397.
- Chen, W. X., Li, G. S., Qi, Y. L., Wang, E. T., Yuan, H. L. & Li, J. L. (1991). *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *Int J Syst Bacteriol* **41**, 275–280.
- Chen, W.-X., Tan, Z.-Y., Gao, J.-L., Li, Y. & Wang, E.-T. (1997). *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *Int J Syst Bacteriol* **47**, 870–873.
- Chen, W.-M., Laevens, S., Lee, T.-M., Coenye, T., De Vos, P., Mergaey, M. & Vandamme, P. (2001). *Ralstonia taiwanensis* sp. nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol* **51**, 1729–1735.
- de Lajudie, P., Willems, A., Pot, B. & 7 other authors (1994). Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. *Int J Syst Bacteriol* **44**, 715–733.
- de Lajudie, P., Willems, A., Nick, G. & 9 other authors (1998a).

Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *Int J Syst Bacteriol* **48**, 369–382.

de Lajudie, P., Laurent-Fulele, E., Willems, A., Torck, U., Coopman, R., Collins, M. D., Kersters, K., Dreyfus, B. & Gillis, M. (1998b). *Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. *Int J Syst Bacteriol* **48**, 1277–1290.

De Ley, J. (1970). Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. *J Bacteriol* **101**, 738–754.

De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.

Dreyfus, B., Garcia, J. L. & Gillis, M. (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int J Syst Bacteriol* **38**, 89–98.

Gao, J. L., Sun, J. G., Li, Y., Wang, E. T. & Chen, W. X. (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan Province, China. *Int J Syst Bacteriol* **44**, 151–158.

Genetics Computer Group (1995). *Program Manual for the Wisconsin Package, Version 8*. Madison, WI: Genetics Computer Group.

Graham, P. H., Sadowsky, M. J., Keyser, H. H. & 8 other authors (1991). Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *Int J Syst Bacteriol* **41**, 582–587.

Jarvis, B. D. W., Pankhurst, C. E. & Patel, J. J. (1982). *Rhizobium loti*, a new species of legume root nodule bacteria. *Int J Syst Bacteriol* **32**, 378–380.

Jarvis, B. D. W., van Berkum, P., Chen, W. X., Nour, S. M., Fernandez, M. P., Cleyet-Marel, J.-C. & Gillis, M. (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int J Syst Bacteriol* **47**, 895–898.

Jordan, D. C. (1982). Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants. *Int J Syst Bacteriol* **32**, 136–139.

Jordan, D. C. (1984). Family III. *Rhizobiaceae* Conn 1938, 321^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–235. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Kuykendall, L. D., Saxena, B., Devine, T. E. & Udell, S. E. (1992). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* **38**, 501–505.

Lindström, K. (1989). *Rhizobium galegae*, a new species of legume root nodule bacteria. *Int J Syst Bacteriol* **39**, 365–367.

Marmur, J. (1961). A procedure for the isolation of DNA from microorganisms. *J Mol Biol* **3**, 208–218.

Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P. & Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* **41**, 417–426.

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the β -subclass of *Proteobacteria*. *Nature* **411**, 948–950.

Nour, S. M., Fernandez, M. P., Normand, P. & Cleyet-Marel, J.-C. (1994). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *Int J Syst Bacteriol* **44**, 511–522.

Nour, S. M., Cleyet-Marel, J.-C., Normand, P. & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *Int J Syst Bacteriol* **45**, 640–648.

Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Rome, S., Fernandez, M. P., Brunel, B., Normand, P. & Cleyet-Marel, J.-C. (1996). *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int J Syst Bacteriol* **46**, 972–980.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Scholla, M. H. & Elkan, G. H. (1984). *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. *Int J Syst Bacteriol* **34**, 484–486.

Segovia, L., Young, J. P. W. & Martínez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. *Int J Syst Bacteriol* **43**, 374–377.

Sneath, P. H. A. & Sokal, R. B. (1973). *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. San Francisco: W. H. Freeman and Co.

Sy, A., Giraud, E., Jourand, P. & 8 other authors (2001). Methylo-trophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. *J Bacteriol* **183**, 214–220.

Tan, Z.-Y., Xu, X.-D., Wang, E.-T., Gao, J.-L., Martínez-Romero, E. & Chen, W.-X. (1997). Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. *Int J Syst Bacteriol* **47**, 874–879.

Tan, Z. Y., Wang, E. T., Peng, G. X., Zhu, M. E., Martínez-Romero, E. & Chen, W. X. (1999). Characterization of bacteria isolated from wild legumes in the north-western regions of China. *Int J Syst Bacteriol* **49**, 1457–1469.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Tiesman, J. & Rizzino, A. (1991). A rapid and reliable method for the purification of high-quality plasmid DNA for double-stranded sequencing. *Biotechniques* **10**, 319, 326–328.

van Berkum, P., Beyene, D., Bao, G., Campbell, T. A. & Eardly, B. D. (1998). *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. *Int J Syst Bacteriol* **48**, 13–22.

Vincent, J. M. (1970). *A Manual for the Practical Study of Root Nodule Bacteria*. Oxford: Blackwell Scientific.

Wang, E. T. & Martínez-Romero, E. (2000). *Sesbania herbacea*–*Rhizobium huautlense* nodulation in flooded soils and comparative characterization of *S. herbacea*-nodulating rhizobia in different environments. *Microb Ecol* **40**, 25–32.

Wang, E. T., van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X. & Martínez-Romero, E. (1998). *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int J Syst Bacteriol* **48**, 687–699.

Wang, E. T., van Berkum, P., Sui, X. H., Beyene, D., Chen, W. X. & Martínez-Romero, E. (1999). Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov. *Int J Syst Bacteriol* **49**, 51–65.

Wieslander, L. (1979). A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Anal Biochem* **98**, 305–309.

Willems, A. & Collins, M. D. (1993). Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *Int J Syst Bacteriol* **43**, 305–313.

Xu, L. M., Ge, C., Cui, Z., Li, J. & Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* **45**, 706–711.

Yanagi, M. & Yamasato, K. (1993). Phylogenetic analysis of the family *Rhizobiaceae* and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiol Lett* **107**, 115–120.

Yelton, M. M., Yang, S. S., Edie, S. A. & Lim, S. T. (1983). Characterization of an effective salt-tolerant, fast-growing strain of *Rhizobium japonicum*. *J Gen Microbiol* **129**, 1537–1547.