Shinella kummerowiae sp. nov., a symbiotic bacterium isolated from root nodules of the herbal legume *Kummerowia stipulacea*

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Bacterial strain CCBAU 25048^T was isolated from root nodules of *Kummerowia stipulacea* grown in Shandong province of China. Cells of the strain were Gram-negative, strictly aerobic, non-spore-forming, motile short rods. Phylogeny of 16S rRNA gene sequences revealed that the strain belonged to the genus *Shinella*, a member of family *Rhizobiaceae*. Its closest phylogenetic relatives were *Shinella granuli* Ch06^T and *Shinella zoogloeoides* IAM 12669^T, respectively showing 98.3 and 98.9 % 16S rRNA gene sequence similarity. Strain CCBAU 25048^T had DNA–DNA relatedness of 43.5 and 34.8 %, respectively, with *S. zoogloeoides* JCM 20728^T and *S. granuli* JCM 13254^T. In addition, in TP-RAPD analysis, different patterns were obtained for these three strains and some rhizobial strains. The *nifH*, *nodC* and *nodD* sequences of CCBAU 25048^T were identical or very similar to those of bean-nodulating *Rhizobium tropici* strains. Several phenotypic characteristics, including the use of citrate and D-ribose as carbon sources and growth at pH 11.0, as well as the fatty acid composition, could differentiate CCBAU 25048^T from the two defined *Shinella* species. Therefore, a novel species *Shinella kummerowiae* sp. nov. is proposed, with strain CCBAU 25048^T (=JCM 14778^T =LMG 24136^T) as the type strain.

Rhizobia are soil bacteria capable of eliciting nodules on leguminous plant roots and/or stems in which the bacteria fix nitrogen. In addition to species in the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Ensifer* (former *Sinorhizobium* species), symbiotic strains belonging to a wide range of bacterial genera have been reported recently from root nodules of several legume species, including *Devosia neptuniae* (Rivas *et al.*, 2002), *Methylobacterium nodulans* (Sy *et al.*, 2001), *Ochrobactrum lupini* (Trujillo *et al.*, 2005) and *Ochrobactrum cytisi* (Zurdo-Piñeiro *et al.*, 2007) in the *Alphaproteobacteria*, plus several species of *Ralstonia* (Chen *et al.*, 2001) and *Burkholderia* (Moulin *et al.*, 2000; Vandamme *et al.*, 2002) in the *Betaproteobacteria*. Meanwhile, some non-symbiotic bacteria related to symbiotic species have also been isolated from root nodules (Chou *et al.*, 2007; Trujillo *et al.*, 2006).

Abbreviation: TP-RAPD, two-primers randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCBAU 25048^T is EF070131.

A comparison of *nifH*, *nodC* and *nodD* sequences and phylogenetic trees based on these sequences are available as supplementary material with the online version of this paper.

In a survey of *Kummerowia* rhizobia (Lin *et al.*, 2007), we isolated strain CCBAU 25048^T from a root nodule of *Kummerowia stipulacea* grown in Shandong province of China. This strain was related only distantly to *Rhizobium giardinii* and was quite different from the defined species of *Bradyrhizobium*, *Rhizobium* and *Ensifer* in the 16S rRNA gene phylogeny (Lin *et al.*, 2007). Therefore, it might represent a novel lineage of symbiotic bacteria. With the aim of clarifying the taxonomic position of this strain, we made a serial of analyses, including reconstruction of the 16S rRNA gene phylogenetic tree by adding the recently defined *Shinella* species, detection and sequencing of symbiotic genes from CCBAU 25048^T, DNA–DNA hybridization, two-primers randomly amplified polymorphic DNA (TP-RAPD) analysis and phenotypic characterization.

The 16S rRNA gene sequence of strain CCBAU 25048^T (Lin *et al.*, 2007) was closely related to those of *Shinella* species, recently described members of the family *Rhizobiaceae* (An *et al.*, 2006), in the phylogenetic tree of 16S rRNA gene sequences (Fig. 1) reconstructed with the neighbour-joining method and bootstrapped with 1000 replications using MEGA program version 3.1 (Kumar *et al.*, 2004). In this tree, CCBAU 25048^T was most similar to *Shinella*

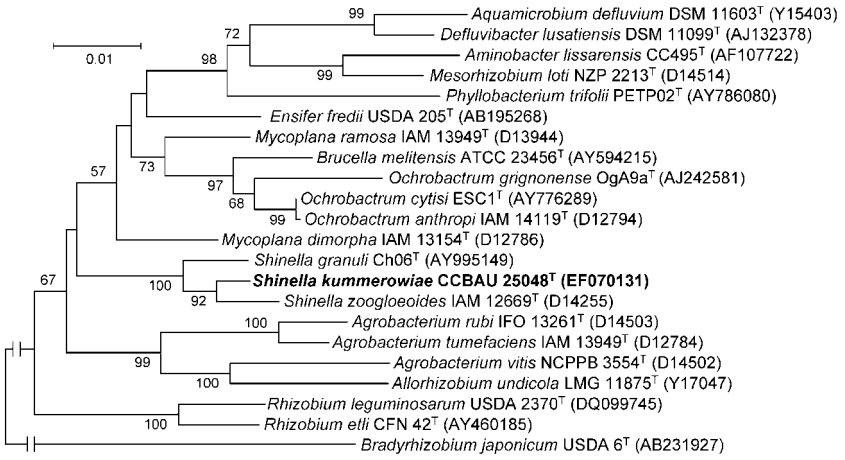

Fig. 1. Neighbour-joining tree reconstructed with 16S rRNA gene sequences showing the phylogenetic relationships of strain CCBAU 25048^T (*Shinella kummerowiae* sp. nov.) and related species. Bootstrap values obtained from 100 pseudoreplicates are provided at the corresponding nodes. Bar, 1 % sequence difference.

granuli Ch06^T (98.3 % sequence similarity), isolated from an upflow anaerobic sludge blanket reactor (An *et al.*, 2006), and to *Shinella zoogloeoides* IAM 12669^T (formerly a strain of *Zoogloea ramigera*) (98.9 % similarity), which produces a characteristic gelatinous matrix of finger-like projections, the so-called 'zoogloeal matrix' (Dugan *et al.*, 1992). These three strains formed a clade and were further linked to *Mycoplana*, *Brucella–Ochrobactrum* and *Agrobacterium* species, which are pathogens for humans or plants (Fig. 1). Lower 16S rRNA gene sequence similarities of 94–96 % were found between strain CCBAU 25048^T and species of *Rhizobium*, *Ensifer* and *Mesorhizobium*. Therefore, CCBAU 25048^T could be identified as a member of *Shinella*, since the 16S rRNA gene sequence phylogeny has been used as the basal criterion to define the bacterial genus.

It has been reported that bacterial strains that show identical patterns in TP-RAPD analysis belong to the same species, whereas different species have distinct patterns (Rivas *et al.*, 2001). In the present study, strain CCBAU 25048^T, *S. granuli* JCM 13254^T, *S. zoogloeoides* JCM 20728^T, *Rhizobium etli* CFN 42^T, *Rhizobium leguminosarum* USDA 2370^T, *Ensifer meliloti* USDA 1002^T and *Mesorhizobium loti* NZP 2213^T were incubated in YMA medium (Vincent, 1970) at 28 °C. DNA extracted from these strains with the method of Terefework *et al.* (2001) was used in TP-RAPD analysis as described previously (Trujillo *et al.*, 2005). The patterns acquired (Fig. 2) contained a band corresponding to the 16S rRNA gene and several others produced by random amplification of genomic DNA. The TP-RAPD pattern of strain CCBAU 25048^T was obviously different from patterns of *S. granuli* JCM 13254^T, *S. zoogloeoides* JCM 20728^T and other rhizobial species.

High-quality DNA was extracted as described previously (Johnson, 1985a, b) from strain CCBAU 25048^T, *S. zoogloeoides* JCM 20728^T and *S. granuli* JCM 13254^T. A DNA G+C content of 63.1 mol% was determined for strain CCBAU 25048^T with the spectrophotometric method (Marmur & Doty, 1962). DNA–DNA hybridization with the initial renaturation-rate method (De Ley, 1970) revealed that strain CCBAU 25048^T had 43.5 % DNA relatedness to *S. zoogloeoides* JCM 20728^T and 34.8 % to *S. granuli* JCM 13254^T. In the light of the recommendation of a threshold value of 70 % DNA–DNA relatedness to define a species (Wayne *et al.*, 1987), these results indicated that strain CCBAU 25048^T could be regarded as a member of a genomic species distinct from *S. granuli* and *S. zoogloeoides*.

For analysis of symbiotic genes, DNA extracted from CCBAU 25048^T, *S. granuli* JCM 13254^T and *S. zoogloeoides* JCM 20728^T with the method of Terefework *et al.* (2001) was used in PCR amplification of *nifH* and *nodD* genes with the primers and methods of Rivas *et al.* (2002). PCR products of *nifH* and *nodD* were obtained from CCBAU 25048^T, but not from *S. granuli* JCM 13254^T or *S. zoogloeoides* JCM 20728^T. The PCR products obtained were sequenced directly as in our previous study (Lin *et al.*,

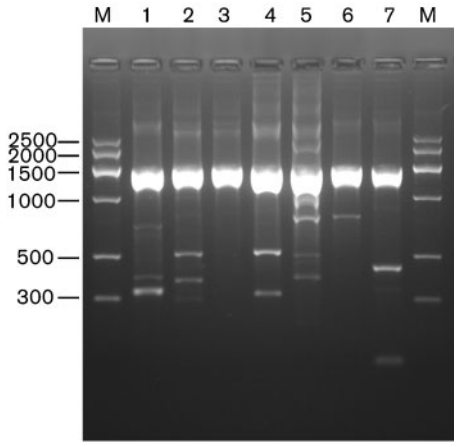

Fig. 2. TP-RAPD profiles showing differences between CCBAU 25048^T (lane 1), *S. zoogloeoides* JCM 20728^T (2) and *S. granuli* JCM 13254^T (3). *R. etli* CFN 42^T (lane 4), *R. leguminosarum* USDA 2370^T (5), *E. meliloti* USDA 1002^T (6) and *M. loti* NZP 2213^T (7) were used as references. M, Molecular DNA markers.

2007) with the same primers. Phylogenetic trees (see Supplementary Fig. S1, available in IJSEM Online) constructed with the same methods used for 16S rRNA gene sequence analysis revealed that the sequences of *nifH* and *nodD* from strain CCBAU 25048^T were very similar (99.2–100 % identity) to those of bean symbionts *Rhizobium tropici* CIAT 899^T and CFN 299 (Martínez-Romero *et al.*, 1991) and *Devosia neptuniae* J1^T, which was isolated from *Neptunia natans* (Rivas *et al.*, 2002). A *nodC* gene sequence identical to those of *R. tropici* CIAT 899^T and CFN 299 (Supplementary Fig. S1) has been reported previously from CCBAU 25048^T (Lin *et al.*, 2007). The *nodC*, *nodD* and *nifH* sequences of strain CCBAU 25048^T showed low identity (43.5–91.6 %) to those of other species of *Rhizobium*, *Bradyrhizobium*, *Ensifer* and *Mesorhizobium*, as well as *Agrobacterium rhizogenes* ATCC 11325^T and *Ochrobactrum lupini* LUP21^T (Supplementary Table S1). These results clearly suggested that the symbiotic genes of CCBAU 25048^T had the same origin as those of bean-nodulating *R. tropici* strains or that this strain might have obtained these symbiotic genes by lateral transfer from nodule-forming bacteria, as reported previously (Sy *et al.*, 2001; Rivas *et al.*, 2002; Trujillo *et al.*, 2005; Moulin *et al.*, 2000; Zurdo-Piñeiro *et al.*, 2007).

Fatty acid profiles have been used previously to distinguish rhizobial species (Tighe *et al.*, 2000; Wang *et al.*, 2007). In this study, the cellular fatty acid composition of CCBAU 25048^T was analysed as described previously (Wang *et al.*, 2007). Seven fatty acid peaks were detected from this strain, corresponding to $C_{14:0}$ (1.28 %), summed feature 1 (4.47 %; $C_{13:0}$ 3-OH and/or iso-$C_{15:1}$ H), $C_{15:0}$ (percentage not calculated), summed feature 2 (44.83 %; $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I), summed feature 3 (6.35 %; $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$ (29.26 %) and $C_{15:0}$ 3-OH (13.8 %). This composition was quite different from those of *Shinella* species (Table 1). With the method of Komagata & Suzuki (1987), the major respiratory quinone in CCBAU 25048^T was determined to be ubiquinone 10 (Q-10), as for the two *Shinella* species (An *et al.*, 2006).

The capacity of CCBAU 25048^T for nodulation was examined by inoculating it to surface-sterilized seeds of *Phaseolus vulgaris*, *Medicago sativa*, *Glycine max*, *Leucaena leucocephala* and its original host, *K. stipulacea*, with the methods of Trujillo *et al.* (2005). After growing for 5 weeks under natural sunlight in a greenhouse, no nodules were observed on any plants inoculated with CCBAU 25048^T, while nodules were observed on roots of *P. vulgaris* inoculated with *R. tropici* CFN 299 (positive control). These results suggested that CCBAU 25048^T may have another host(s) and that it might be a nodule endophyte in *K. stipulacea*, as reported for other bacteria in previous studies (Chou *et al.*, 2007).

Taking the results of 16S rRNA gene sequence phylogeny, TP-RAPD and DNA–DNA hybridization in the present study and our previous results (Lin *et al.*, 2007), it is clear that CCBAU 25048^T represents a novel genomic species within the genus *Shinella*. To verify whether strain CCBAU 25048^T corresponded to a taxonomic species, phenotypic features were analysed and compared with related species.

Table 1. Characteristics useful for discriminating the novel *Shinella* species from other species in the genus

Strains: 1, CCBAU 25048^T (*S. kummerowiae* sp. nov.); 2, *S. granuli* JCM 13254^T; 3, *S. zoogloeoides* JCM 20728^T. +, Positive; –, negative; ND, not done.

Characteristic	1	2	3
Assimilation of:			
Gluconate	+	+	–
Malate	+	+	–
D-Xylose	+	–	+
Salicin	–	+	–
Citrate	+	–	–
D-Ribose	–	+	+
Growth at/in:			
4 % NaCl	–	+	+
pH 5.5	+	–	+
pH 11.0	+	–	–
37 °C	–	+	–
Resistance to: (μg ml^{-1})			
Kanamycin (100)	–	+	ND
Streptomycin (300)	+	–	ND
Ampicillin (300)	+	ND	ND
Fatty acids (%)			
$C_{14:0}$	1.2	–	–
$C_{15:0}$	6.5	–	–
$C_{15:0}$ 3-OH	12.9	–	–
$C_{16:0}$	27.4	9.8–10.4	13.4
$C_{16:0}$ 3-OH	–	1.6–2.2	8.1
$C_{18:0}$	–	1.6–2.6	2.6
$C_{19:0}$ cyclo $\omega8c$	–	3.0–4.9	2.9
Summed feature 1*	4.2	–	–
Summed feature 2*	41.8	–	–
Summed feature 3*	6.0	4.4–5.5	–
Summed feature 7*	–	75.8–76.9	72.8
Presence of:			
nodC/*nodD*	+	–	–
nifH	+	–	–
DNA G+C content (mol%) (T_m)	63.1	66	64

*Summed feature 1 contains $C_{13:0}$ 3-OH and/or iso-$C_{15:1}$ H. Summed feature 2 contains $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I. Summed feature 3 contains $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$. Summed feature 7 contains $C_{18:1}\omega7c/\omega9t/\omega12t$ and/or $C_{18:1}\omega7c/\omega9c/\omega12t$.

To identify distinctive features of CCBAU 25048^T, cellular morphology was observed under an Olympus B07 optical microscope. Utilization of 45 carbon sources and 13 nitrogen sources was determined as described previously (Chen *et al.*, 1988). Resistance to antibiotics was determined by using different final concentrations (5, 50, 100 and 300 μg ml^{-1}) of kanamycin, ampicillin, erythromycin, streptomycin, gentamicin, chloromycin and neomycin on YMA medium (Vincent, 1970). Tolerance of NaCl was

measured on YMA medium containing 1, 3 and 5 % (w/v) NaCl. The pH range for growth was studied between pH 4.0 and 12.0 in the same medium at intervals of 0.5 pH units. Growth at 37 °C on YMA medium and 28 °C on Luria–Bertani (LB) medium was also tested. The strain was Gram-negative, motile in hanging drops and exhibited catalase, oxidase, β-galactosidase and β-glucosidase activities tested with standard procedures (Cowan & Steel, 1970). Distinctive characteristics of strain CCBAU 25048^T in comparison with the two *Shinella* species and some rhizobial species are shown in Table 1. Assimilation of citrate and D-ribose as carbon sources and growth at pH 11.0 differentiate CCBAU 25048^T from the two defined *Shinella* species.

The results obtained in the present and previous studies show that CCBAU 25048^T represents a novel species within the genus *Shinella* which we name *Shinella kummerowiae* sp. nov. The description of this novel species enlarges the breadth of known symbiotic bacteria into the genus *Shinella*.

Description of *Shinella kummerowiae* sp. nov.

Shinella 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 type strain was isolated).

Gram-negative, motile, aerobic, non-spore-forming rods (0.5–0.7 × 1.4–2.0 μm). Colonies are circular, cream coloured, semi-translucent and 2–4 mm in diameter after 3 days incubation at 28 °C on YMA medium. Catalase, oxidase, β-galactosidase and β-glucosidase activities are present. Optimum temperature for growth is 25–30 °C; no growth at 37 °C. Optimum growth at pH 7–8; can grow at pH 5–11. Can grow at 3 % NaCl and on LB medium. No growth in medium containing adipate, malonate, inulin, melibiose, acetate, hippurate, starch, syringate, tartrate, vanillate, D-ribose, salicin or methionine as sole carbon sources. Cells are resistant to ampicillin (300 μg ml^{-1}), erythromycin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}) and streptomycin (300 μg ml^{-1}), but susceptible to chloromycin (5 μg ml^{-1}), neomycin (10 μg ml^{-1}) and gentamicin (50 μg ml^{-1}).

The type strain is CCBAU 25048^T (=JCM 14778^T =LMG 24136^T), which was isolated from root nodules of *Kummerowia stipulacea* grown in Shandong province, China. The G+C content of the type strain is 63.1 mol%. The type strain contains symbiotic genes *nodC*, *nodD* and *nifH* similar to those of *R. tropici* CIAT 899^T and CFN 299.

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