NOTE

***Sinorhizobium meliloti* associated with *Medicago sativa* and *Melilotus* spp. in arid saline soils in Xinjiang, China**Ai Min Yan,¹ En Tao Wang,² Feng Ling Kan,¹ Zhi Yuan Tan,³ Xin Hua Sui,¹ Barbara Reinhold-Hurek³ and Wen Xin Chen¹Author for correspondence: Wen Xin Chen. Tel: +86 10 62891854. Fax: +86 10 62891055.
e-mail: wenxin_chen@263.net¹ College of Biological Sciences, China Agricultural University, Beijing 100094, People's Republic of China² Departamento de Microbiologica, Escuela Nacional de Ciencias Biologicas, Instituto Politecnico Nacional, Prol. de Carpio y Plan de Ayala, 11340 Mexico D. F., Mexico³ Max-Planck-Institut für Terrestrische Mikrobiologie, Arbeitsgruppe Symbioseforschung, D-35043 Marburg, Germany**Of 42 rhizobial isolates from *Medicago sativa* and *Melilotus* spp. growing in arid saline fields in Xinjiang, China, 40 were identified as *Sinorhizobium meliloti* by a polyphasic approach. However, diverse groups were obtained from these isolates in numerical taxonomy and SDS-PAGE of proteins. They could grow at pH 10.5 and were tolerant to 2.5–4.0% (w/v) NaCl.****Keywords:** *Sinorhizobium meliloti*, *Medicago sativa*, *Melilotus* spp., diversity, phylogeny

The legume species within the genera *Medicago* and *Melilotus* are important as foliage and as a source of green manure and are naturally distributed or cultivated in many regions of the world. They are known to form nitrogen-fixing root nodules. *Sinorhizobium meliloti* (de Lajudie *et al.*, 1994), *Sinorhizobium medicae* (Rome *et al.*, 1996) and *Rhizobium mongolense* (van Berkum *et al.*, 1998) have been described as rhizobia associated with these plants. However, compared to the worldwide distribution and large number of species within these two legume genera (Allen & Allen, 1981), the diversity of the rhizobia associated with these legumes has still not been well evaluated.

Many *Medicago* and *Melilotus* species grow widely in China, but their microsymbionts have not been investigated systematically. A few isolates from these plants in Chinese soils have been classified as *S. meliloti* (Chen *et al.*, 1988) and *R. mongolense* (van Berkum *et al.*, 1998). As a foliage crop, *Medicago sativa* has been cultivated for many years in a vast area in Xinjiang, China. Some species of *Melilotus* are native to that

region and provide food for wild animals. Soils are mainly saline and alkaline in this area. It could be reasoned that rhizobia adapted to this environment are needed for effective symbiosis. Considering the potential ability of using salt- and saline-tolerant rhizobia as inoculant to improve the production of alfalfa in arid regions, we collected and characterized the rhizobial germplasms associated with *Medicago sativa* and *Melilotus* species in the arid saline fields of Shihezi, Urumqi and Turpan, three different geographic regions (cities) from north to south in Xinjiang, China.

A total of 42 isolates and 26 reference strains (Table 1) were used in this research. Forty-one isolates originally obtained from the nodules were fast-growing, acid-producing rhizobia with gummy colonies on YMA medium. Since small, dry colonies occurred frequently in many isolates, isolates CCBAU83333S, a small colony derivative of isolate CCBAU83333, was also included in some of the analyses. Numerical taxonomy analysis, using the methods described by Gao *et al.* (1994), divided 38 of the 41 isolates into two clusters (1 and 2) at a similarity level of 85% and they linked together at 82% similarity before they linked to the defined species (Fig. 1). Isolates from the two genera of host plants in the three geographic regions were intermingled in these two clusters. Three isolates,

Abbreviations: ET, electrophoretic type; MLEE, multilocus enzyme electrophoresis.

The GenBank accessions number for the 16S rRNA gene sequences of strains CCBAU83060 and CCBAU83408 are AF054930 and AF054931, respectively.

Table 1. Bacteria used in this research

Isolate or strain*	Host plant	Geographic origin	Source or reference
Cluster 1			
CCBAU83060–83062, 83065, 83067–83071, 83073, 83077, 83086, 83090, 83095, 83097, 83100, 83103	<i>Medicago sativa</i>	Turpan†	CCBAU
CCBAU83483, 83489, 83542, 83552	<i>Medicago sativa</i>	Shihezi†	CCBAU
CCBAU83310	<i>Medicago sativa</i>	Urumqi†	CCBAU
CCBAU83328	<i>Melilotus albus</i>	Shihezi†	CCBAU
CCBAU83322, 83342	<i>Melilotus suaveolens</i>	Shihezi†	CCBAU
CCBAU83700	<i>Melilotus</i> sp.	Shihezi†	CCBAU
CCBAU83420	<i>Melilotus suaveolens</i>	Urumqi†	CCBAU
CCBAU83394, 83382	<i>Melilotus</i> sp.	Urumqi†	CCBAU
Cluster 2			
CCBAU83075, 83092, 83099	<i>Medicago sativa</i>	Turpan†	CCBAU
CCBAU83318, 83321, 83336	<i>Melilotus albus</i>	Shihezi†	CCBAU
CCBAU83402, 83408	<i>Melilotus suaveolens</i>	Urumqi†	CCBAU
CCBAU83370	<i>Melilotus</i> sp.	Shihezi†	CCBAU
Other isolates			
CCBAU83333, 83335	<i>Melilotus</i> sp.	Shihezi†	CCBAU
CCBAU83482, 83557	<i>Medicago sativa</i>	Shihezi†	CCBAU
Reference strains			
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> USDA2370 ^T	<i>Pisum sativum</i>	USA	CCBAU
<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i> 127K17	<i>Phaseolus vulgaris</i>	USA	CCBAU
<i>Rhizobium etli</i> CFN42 ^T	<i>Phaseolus vulgaris</i>		Segovia <i>et al.</i> (1993)
<i>Rhizobium galegae</i> HAMBI540 ^T	<i>Galega orientalis</i>	Finland	
<i>Rhizobium galegae</i> HAMBI1185	<i>Galega</i> sp.	USA	Lindstrom (1989)
<i>Rhizobium tropici</i> CIAT899 ^T	<i>Phaseolus vulgaris</i>	Columbia	Martinez-Romero <i>et al.</i> (1991)
<i>Rhizobium tropici</i> BR853	<i>Leucaena leucocephala</i>	Brazil	Martinez-Romero <i>et al.</i> (1991)
<i>Rhizobium hainanense</i> CCBAU57015 ^T (166 ^T)	<i>Desmodium sinuatum</i>	Hainan†	Chen <i>et al.</i> (1997)
<i>Rhizobium hainanense</i> H14	<i>Desmodium heterophyllum</i>	Hainan†	Chen <i>et al.</i> (1997)
<i>Rhizobium mongolense</i> USDA 1844 ^T	<i>Medicago ruthenica</i>	Inner Mongolia†	van Berkum <i>et al.</i> (1998)
<i>Rhizobium giardinii</i> USDA2914 ^T (H 152 ^T)	<i>Phaseolus vulgaris</i>	France	Amarger <i>et al.</i> (1997)
<i>Rhizobium gallicum</i> USDA2918 ^T (R602sp ^T)	<i>Phaseolus vulgaris</i>	France	Amarger <i>et al.</i> (1997)
<i>Sinorhizobium fredii</i> USDA205 ^T , USDA194	<i>Glycine soja</i>	Henan†	Scholla <i>et al.</i> (1984)
<i>Sinorhizobium teranga</i> USDA4101 ^T (LMG7834 ^T)	<i>Acacia laeta</i>	Senegal	de Lajudie <i>et al.</i> (1994)
<i>Sinorhizobium saheli</i> USDA4102 ^T (LMG7837 ^T)	<i>Sesbania cannabine</i>	Senegal	de Lajudie <i>et al.</i> (1994)
<i>Sinorhizobium meliloti</i> USDA1002 ^T , 102F28	<i>Medicago sativa</i>	USA	CCBAU
<i>Sinorhizobium medicae</i> USDA1037 ^T (A321 ^T)	<i>Medicago</i> spp. (annual)	France	More <i>et al.</i> (1996)
<i>Sinorhizobium xinjiangense</i> CCBAU110 ^T	<i>Glycine max</i>	Xinjiang†	Chen <i>et al.</i> (1988)
<i>Mesorhizobium huakuii</i> CCBAU2609 ^T	<i>Astragalus sinicus</i>	Hubei†	Chen <i>et al.</i> (1991)
<i>Mesorhizobium huakuii</i> A106	<i>Astragalus sinicus</i>	Nanjing†	Chen <i>et al.</i> (1991)
<i>Mesorhizobium loti</i> NZP2213 ^T	<i>Lotus corniculatus</i>		
<i>Mesorhizobium loti</i> NZP2227	<i>Lotus</i> sp.	New Zealand	Jarvis <i>et al.</i> (1982)
<i>Mesorhizobium tianshanense</i> CCBAU3306 ^T (A1BS ^T)	<i>Glycyrrhiza pallidiflora</i>	Xinjiang†	Chen <i>et al.</i> (1995)
<i>Mesorhizobium tianshanense</i> 6	<i>Glycyrrhiza uralensis</i>	Xinjiang†	Chen <i>et al.</i> (1995)
<i>Mesorhizobium ciceri</i> USDA3378 ^T (UPM-Ca7 ^T)	<i>Ciceri arietinum</i>	Mediterranean basin	Nour <i>et al.</i> (1994)

* CCBAU, Culture Collection of Beijing Agriculture University, Beijing, China; USDA, Beltsville *Rhizobium* Culture Collection, Beltsville Agricultural Research Center, US Department of Agriculture, Beltsville, MD, USA; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, K.-L. Ledeganckstraat, 35, B-9000 Ghent, Belgium; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; CIAT, *Rhizobium* Collection, Centro Internacional de Agricultura Tropical, Cali, Columbia; CFN, Centro de Investigacion sobre Fijacion de Nitrogeno, UNAM, Cuernavaca, Morelos, Mexico; NZP, Division of Scientific and Industrial Research, Palmerston North, New Zealand.

† City or Province of China.

CCBAU83333, 83557 and 83482 were not grouped into any cluster. Four groups at a similarity level of 81 % were obtained in a cluster analysis of SDS-PAGE protein patterns using the methods of Tan *et al.* (1997) (results not shown). Isolates within each of the two clusters were separated into different groups and some isolates from both clusters were intermingled into two of the four groups. A single isolate, CCBAU83333, was grouped together with isolates from cluster 1.

Using the methods of Wang *et al.* (1998) and the restriction endonucleases *Msp*I, *Hinf*I, *Hha*I and *Sau*3AI, identical PCR-amplified 16S rRNA gene RFLP patterns were obtained from 12 isolates rep-

resenting clusters 1 and 2 and from *S. meliloti* USDA 1002^T and *S. medicae* USDA 1037^T (not shown). Isolate CCBAU83333 and its small colony derivative, CCBAU83333S, also shared identical patterns with other isolates. Isolate CCBAU83482 had unique 16S rRNA gene RFLP patterns different from all defined *Sinorhizobium* and *Rhizobium* species, but most similar to those of *Rhizobium mongolense* and *Rhizobium gallicum* (patterns not shown). The full 16S rRNA gene sequences obtained by a procedure of directly sequencing the PCR products (Hurek *et al.*, 1997) from isolates CCBAU83060, representing cluster 1, and CCBAU83099, representing cluster 2, were identical to that of *S. meliloti* USDA 1002^T.

In DNA–DNA hybridization, performed according to the method of De Ley *et al.* (1970), isolates within clusters 1 and 2 formed a single genomic species sharing DNA relatedness ranging from 71.7 to 98.7% and they shared DNA homologies of 78.1–100% with *S. meliloti* USDA 1002^T. DNA homologies among representative isolates of clusters 1 and 2 and type strains for other species within the genera *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* (Table 1) ranged from 0 to 39.3%. The close relationships among our isolates and *S. meliloti* were also supported by the results of multilocus enzyme electrophoresis (MLEE). Based on the combination of electrophoretic patterns of aconitase, adenylate kinase, alanine dehydrogenase, esterase, glucose-6-phosphate dehydrogenase, NADP-glutamate dehydrogenase, hexokinase, isocitrate dehydrogenase, indophenol oxidase, malate dehydrogenase, malic enzyme, phosphoglucose isomerase and phosphoglucomutase, seven electrophoretic types (ETs) were identified among the isolates tested (Fig. 2). Isolates of clusters 1 and 2 (ET1–5) were classified into a single group together with the type strain *S. meliloti* USDA 1002^T at a genetic distance of 0.20 (Fig. 2). Single branches that separated above a genetic distance of 0.40 were obtained from isolates CCBAU83482 and 83328, and from the type strains of *Sinorhizobium fredii*, *Sinorhizobium xinjiangense*, *Sinorhizobium medicae*, *Sinorhizobium saheli* and *Sinorhizobium terangae*. Isolate CCBAU83333 had identical MLEE patterns with its mother isolate, CCBAU83333 and they were grouped together with isolates of clusters 1 and 2.

In current taxonomy, DNA–DNA homology and distinctive features are used as the main basis to define a species, and other methods to reveal the genetic diversity are recommended (Graham *et al.*, 1991). The high DNA homologies (> 70%) in DNA–DNA hybridization, close genetic relationships in MLEE analysis (Fig. 2) and identical 16S rRNA gene sequences found among our isolates and the reference strains for *S. meliloti* indicated that the isolates within clusters 1 and 2 (Fig. 1), as well as isolate CCBAU-83333, were *S. meliloti* based on the current definition of species (Graham *et al.*, 1991; Wayne *et al.*, 1987). Isolates CCBAU83060 and 83408, representing clusters 1 and 2, respectively, nodulated *Medicago sativa* and *Melilotus suaveolens*, but did not nodulate *Vigna sinensis*, *Glycine max*, *Trifolium repens*, *Phaseolus vulgaris*, *Pisum sativum* or *Caragana korshinskii*.

It seems there was a lack of *S. medicae* (Rome *et al.*, 1996) in the fields we investigated. However, the identification of isolate CCBAU83482 might indicate the existence of new lineage related to *R. mongolense* (van Berkum *et al.*, 1998) as a minor constituent.

Fig. 1. Dendrogram showing the results of numerical taxonomy based upon 134 phenotypic characteristics. Cluster analysis was performed using unweighted average linkage (Sneath & Sokal, 1973).

Fig. 2. Genetic relationships revealed by MLEE analysis based upon 13 metabolic enzymes. Genetic distance (D) was estimated by the proportion of different enzyme bands among the total bands as described by Selander *et al.* (1986). Cluster analysis was performed using the methods of Nei & Li (1979). ET2 also includes the isolates CCBAU83333, 83333S, 83342, 83420 and 83552; ET4 also includes the isolates CCBAU83060 and 83318; ET5 also includes the isolates CCBAU83071 and 83834.4.

In this study, the grouping results from numerical taxonomy data and from SDS-PAGE of proteins were different from each other and from those of DNA–DNA relatedness, MLEE and 16S rRNA gene analyses. These results further demonstrate that a poly-phasic approach is needed for a stable and accurate classification. The three phenotypic groups obtained among the *S. meliloti* isolates and strains by numerical taxonomy might be evidence for divergent evolution within the species. The new isolates could be differentiated from the reference strains of *S. meliloti* by utilization of D-galactose and trehalose as carbon source, but not gluconate, resistance to 300 µg erythromycin ml⁻¹ and sensitivity to 300 µg polymyxin ml⁻¹, and growth at pH 10.0–10.5 and in medium with 2.5% (w/v) NaCl.

Because *Medicago sativa* is an important foliage and is cultivated worldwide, the microsymbionts associated with this plant have been investigated extensively. It has been reported that *S. meliloti* grows at a pH range of 5.0–9.5 (Jordan, 1984) and is tolerant to 2.0% (w/v) NaCl. Another *Medicago*-nodulating species, *S. medi-*

cae, can grow at pH 5.0–10.0 and is resistant to 2.0% NaCl (Rome *et al.*, 1996). Growth at pH 5.0 has been recorded for only some strains (Jordan, 1984) from acidic soils (del Papa *et al.*, 1999). Our results indicated that the *S. meliloti* isolates from arid-saline areas in Xinjiang were tolerant to high pH and high concentrations of NaCl and were sensitive to acidic conditions. All of them could grow at pH 10.0–10.5, but none could grow at pH 5.0 and only six could grow at pH 5.5. All of them were tolerant to 2.5% (w/v) NaCl. Isolates CCBAU83103, 83062, 83328, 83370, 83382, 83394, 83420, 83482 and 83483 were tolerant to 3% (w/v) NaCl. Isolates CCBAU83328, 83370, 83382, 83420, 83482 and 83483 were even tolerant to 4% (w/v) NaCl. Based on these results, the description of the pH range for growth and resistance to NaCl for *S. meliloti* should be modified. We also found in this research that all of the isolates had gummy colonies on YMA, as for *S. medicae* strains. After checking the isolate CCBAU83333S by PCR-RFLP of 16S rRNA genes and MLEE analysis, we concluded that the small colonies that occurred in many isolates are mutants from the gummy colonies.

Acknowledgements

This research is supported by China–EC collaboration project (No. IC18-CT96-0103) and by the National Natural Science Foundation of China (Nos 39730010 and 39670017).

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