A *Rhizobium* strain that nodulates and fixes nitrogen in association with alfalfa and soybean plants

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A *Rhizobium meliloti* strain that forms effective nodules on soybean (*Glycine max*) and alfalfa (*Medicago sativa*) is described. The strain, 042B, was isolated from root nodules of alfalfa in Xinjiang Autonomous Region of China. Experiments showed that strain 042B was able to nodulate soybean as effectively as *Bradyrhizobium japonicum* USDA110, a widely used inoculant strain. Under hydroponic conditions, both strains performed similarly in many respects such as time required for the appearance of nodules, total nitrogenase activity, plant top dry weight and total plant nitrogen. In soil pot experiments, total nitrogen, seed weight and seed nitrogen of soybean plants inoculated with strains 042B or USDA110 were much higher than those of the uninoculated control, but there were no differences between plants with strain 042B relative to strain USDA110. However, when strain 042B was the inoculant, the number of nodules was greater, but the nodules were smaller than those of strain USDA110. Results from ELISA showed that nodule occupancy of strain 042B in soybean ranged from 82–90% and that of strain USDA110 ranged from 78–86%. Strain 042B can grow in the presence of 5% (w/v) NaCl, at 42 °C, and at pH 10.7. Strain 042B was shown to have two large plasmids (molecular sizes 200 kb and > 1000 kb, respectively). The DNA G+C mol% of strain 042B was 63.6. The DNA level of homology between strain 042B and type-strain *R. meliloti* USDA1002 was 76%, while DNA homologies of strain 042B were 10% and 12% with *R. fredii* USDA205 and *B. japonicum* USDA6, respectively.

Keywords: *Rhizobium meliloti*, symbiotic nitrogen fixation, broad host range

INTRODUCTION

Rhizobium strains form nodules only on a restricted number of legume plants, and each legume host is nodulated only by a limited number of microsymbionts (Jordan, 1984). However, the range of host specificity varies greatly among rhizobia. For instance, *Rhizobium* sp. strain NGR234 nodulates more than 60 different legume genera and the non-legume *Parasponia* (Denarie *et al.*, 1993). In contrast, *Rhizobium meliloti* normally has a rather restricted host range, being symbiotic only with species of *Medicago*, *Melilotus* and *Trigonella* (Jordan, 1984). In recent years phylogenetic studies of rhizobia have shown that *R. meliloti* has a very close relationship with the fast-growing soybean rhizobium, *R. fredii*; 16S rRNA sequences encoded by a 260 bp segment of the DNA from these two species were identical (Jarvis *et al.*, 1992). A close phylogenetic relationship is also supported by serology (Sadowsky *et al.*, 1987). Eardly *et al.* (1992) reported that

strain Or 191 (identified as a relative of *R. etli*) nodulated both alfalfa and common bean, but in that case nitrogen fixation was poor.

The symbiotic efficiency of *R. meliloti* and legume host is high, yielding more fixed nitrogen per hectare per year than other rhizobia including soybean rhizobia (Button, 1980). *R. meliloti* has some specific free-living growth characteristics, such as high salt tolerance (Chen *et al.*, 1987), an optimum growth temperature of 35 °C, with some strains growing at 42 °C (Jordan, 1984), and a generation time of 2 h (Vincent, 1982).

Strain 042B was isolated from root nodules of alfalfa in Wulumuqi when a survey of rhizobial resource was made in Xinjiang Autonomous Region of China in 1980 (Chen *et al.*, 1987). We used strain 042B as a donor in conjugation experiments, aimed at constructing salt-tolerant highly effective strains of soybean rhizobia. When strain 042B was inoculated on soybean as a control in plant nodulation

experiments, it was unexpectedly found to nodulate and fix nitrogen with soybean. In this paper, we describe some basic symbiotic, physiological and biochemical characteristics of strain 042B, and its taxonomic relatedness to other rhizobia.

METHODS

Strains. The bacterial strains used in this study are listed in Table 1.

Media. YMA medium used to cultivate rhizobia was described by Vincent (1970), YM liquid medium is YMA medium without agar. TY liquid medium [0.5% (w/v) Difco Bacto-Tryptone, 0.3% (w/v) Difco Bacto-Yeast Extract and 7 mM CaCl_2] was used to cultivate rhizobia. The hydrophobic medium was prepared according to Zhou *et al.* (1979) and was used to cultivate soybean and, after adding 1% (w/v) agar to make slopes, for nodulation experiments with alfalfa.

Reisolation of rhizobia. Ten nodules were randomly selected from both alfalfa and soybean plants separately inoculated with strain 042B, immersed in 95% (v/v) ethanol for 3 min and subjected to 0.1% HgCl_2 for 5 min. Nodules were rinsed in six changes of sterile water, and homogenized in 2 ml sterile water. Serial 10-fold dilutions of the nodule homogenate were made and 0.1 ml of the dilutions was spread on YMA agar plates containing 0.25 mg bromothymol blue l^{-1} . After surface sterilization, the sterile water used for rinsing the nodules was streaked on YMA agar plates to verify the sterility of the water. All plates were incubated at 28 °C for 7 d. For each nodule isolate a single colony was selected and restreaked on YMA agar plates and checked for purity. Once pure cultures had been confirmed, each isolate was streaked on YMA agar slopes for nodulation experiment.

Plant nodulation experiments. Nodulation experiments on soybean and alfalfa were carried out using the method described by Vincent (1970) with some modification (Chen *et al.*, 1991). Jingyin No. 1 soybean was grown in jars using a hydroponic

method. The nutrient solution used for the plants contained, per litre of distilled water: 0.03 g $\text{Ca}(\text{NO}_3)_2$, 0.46 g CaSO_4 , 0.06 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.14 g K_2HPO_4 , 0.07 g KCl, 0.07 g iron citrate and 1 ml of a trace element solution. The trace element solution contained, per litre of distilled water: 2.86 g H_3BO_3 , 1.8 g MnSO_4 , 0.8 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2 g ZnSO_4 and 0.02 g HMoO_4 . Baoding alfalfa was grown in sterilized vermiculite in glass tubes (200 × 30 mm) plugged with cotton wool containing the same nutrient solution and nitrogenase activities were determined by acetylene reduction assays (Hardy *et al.*, 1973).

Soil pot experiments. Soil pot experiments were done without sterilization in field soil which had been in soybean production. The experiments included inoculation with strain USDA110, inoculation with strain 042B and a control without inoculation, with 12 separate pots for each. All the pots were placed on the ground in the field. Assays of the nitrogenase activity of root nodules, plant top dry weight and total nitrogen were determined at the flowering stage (50 d), the pod stage (62 d) and the seed stage (72 d), with three replicates for each. In the harvest stage (102 d), seeds were collected from the final three pots for determination of grain yield and total nitrogen.

Agglutination. Antigens were prepared from cells grown in TY liquid medium for 2 d. Somatic cell antigens for injections and for agglutination studies were prepared by heating cell suspensions in a boiling water bath for 30 min. Boiled cell suspensions were used to produce somatic cell antisera by the method of Schmidt *et al.* (1968). Tube agglutination reactions were performed essentially as described by Vincent (1970).

Nodule occupancy determined by ELISA. ELISA for identification of the soybean rhizobia strain from nodules was performed as described by Fuhrmann & Wollum (1985).

Growth characteristics. Growth rates at different pH values were determined as described by Yang & Li (1988). Salt tolerance was determined by adding 0.5, 1, 2, 3, 4, 5 and 6% (w/v) NaCl to YMA medium. A growth response in the presence of NaCl was scored as positive if a strain grew as well as, or better than, it did on control plates containing no additional NaCl. The growth responses of given strains were tested on YMA at different temperatures to determine the maximum temperature at which bacterial growth could take place.

Plasmid analysis. Plasmid analysis was carried out by the method of Eckhardt (1978).

Determinations of DNA G + C mol%. DNA was prepared by the method of Johnson (1985). The mean DNA G + C mol% of each strain was measured using the thermal denaturation method (Marmur & Doty, 1962) and the equation of De Ley (1970). *Escherichia coli* K12 was used as the standard.

DNA homology. DNA samples were treated using the method of De Ley *et al.* (1970) and the levels of homology were determined using the initial renaturation rate method (De Ley *et al.*, 1970).

RESULTS

Strain 042B nodulates and fixes nitrogen on both alfalfa and soybean

The following tests demonstrated that strain 042B was indeed a strain that could nodulate both alfalfa and soybean. When strain 042B reached mid-exponential growth phase in TY liquid medium, a series of dilutions was made with physiological saline containing 0.01% Tween 20 and then spread on YMA plates to isolate single colonies. Twenty single colonies were randomly picked

Table 1. Bacterial strains

Strain	Source*
<i>Rhizobium meliloti</i> group A	
042B	CCBAU
41	CAAS
USDA 1002 ^T	USDA
102F28	USDA
CC2013	B. D. Eardly
<i>R. meliloti</i> group B	
CC169	B. D. Eardly
<i>Bradyrhizobium japonicum</i>	
USDA110	USDA
USDA 6 ^T	USDA
<i>Rhizobium fredii</i>	
RT19	This laboratory
USDA 205 ^T	USDA
CCBAU110	CCBAU

* CAAS, Chinese Academy of Agricultural Sciences; CCBAU, Culture Collection of Beijing Agricultural University; USDA, United States Department of Agriculture. B. D. Eardly, Pennsylvania State University, Berks Campus, Reading, PA, USA.

Table 2. Hydroponic nodulation experiment with strains 042B and USDA110 and soybean plants

The values are means of six soybean plants $\pm$ SE. The superscripts a and b indicate statistical similarity and dissimilarity in pairwise comparison.

Strain	Time of appearance of nodules (d)	Nodule number	Nodule wt (mg)	Total nitrogenase activity (nmol C ₂ H ₄ h ⁻¹)	Plant top dry wt (mg)	Total nitrogen (mg)
042B	10	106 ^a $\pm$ 18	583 ^a $\pm$ 86	22 445 ^a $\pm$ 1251	1322 ^a $\pm$ 126	42.15 ^a $\pm$ 7.02
USDA110	10	63 ^b $\pm$ 15	636 ^a $\pm$ 65	20 889 ^a $\pm$ 1330	1362 ^a $\pm$ 131	34.69 ^a $\pm$ 5.14
Uninoculated	—	—	—	—	995 ^b $\pm$ 128	9.89 ^b $\pm$ 5.04

Table 3. Soil pot experiment with strains 042B and USDA110 analysed in growing stages of soybean plants

The values are means of three replicates $\pm$ SE. Superscripts a and b indicate statistical similarity and dissimilarity in pairwise comparison.

Growing stage	Strain	Nodule number	Nodule wt (mg)	Total nitrogenase activity (nmol C ₂ H ₄ h ⁻¹)	Plant top dry wt (g)	Total nitrogen (%)
Flowering stage	042B	266 $\pm$ 38	4827 $\pm$ 525	23 111 ^a $\pm$ 1623	33.49 ^a $\pm$ 3.02	3.65 ^a $\pm$ 0.27
	USDA110	241 $\pm$ 26	3883 $\pm$ 491	28 333 ^a $\pm$ 1425	27.62 ^b $\pm$ 2.87	3.63 ^a $\pm$ 0.22
	Uninoculated	164 $\pm$ 35	4490 $\pm$ 562	17 778 ^b $\pm$ 1990	27.77 ^b $\pm$ 3.12	3.35 ^b $\pm$ 0.31
Pod stage	042B	327 $\pm$ 42	7600 $\pm$ 618	148 161 ^a $\pm$ 3836	43.59 ^a $\pm$ 2.11	3.64 ^a $\pm$ 0.31
	USDA110	238 $\pm$ 38	6830 $\pm$ 520	78 000 ^b $\pm$ 3235	37.35 ^b $\pm$ 3.59	3.41 ^a $\pm$ 0.23
	Uninoculated	315 $\pm$ 45	10 617 $\pm$ 735	79 556 ^b $\pm$ 4250	48.26 ^a $\pm$ 3.88	2.95 ^b $\pm$ 0.25
Seed stage	042B	296 $\pm$ 41	7187 $\pm$ 535	34 445 ^a $\pm$ 1940	51.34 ^a $\pm$ 7.38	3.72 ^a $\pm$ 0.22
	USDA110	213 $\pm$ 31	6573 $\pm$ 581	32 333 ^a $\pm$ 2201	51.34 ^a $\pm$ 6.08	3.56 ^a $\pm$ 0.28
	Uninoculated	347 $\pm$ 37	9677 $\pm$ 620	32 555 ^a $\pm$ 1629	53.80 ^a $\pm$ 6.68	3.10 ^b $\pm$ 0.32

and inoculated separately onto 20 soybean plants cultured in hydroponic medium and 20 alfalfa plants cultured on the same medium with 1 % agar as slopes. After 10 d, all the soybean and alfalfa plants were nodulated and had acetylene-reducing activity. In contrast, *B. japonicum* USDA110 could only nodulate and fix nitrogen with soybean, and *R. meliloti* USDA1002 could only do so with alfalfa. Blank controls for both plants were not nodulated.

The isolates obtained from soybean root nodules inoculated with strain 042B were fast-growing rhizobia. When these isolates were used to inoculate alfalfa and soybean both plants were nodulated and fixed nitrogen. The isolates obtained from alfalfa root nodules inoculated with strain 042B had similar nodulation and nitrogen fixation characteristics.

DNA homology was determined between strain 042B, the re-isolates obtained from soybean root nodule, and the re-isolates obtained from alfalfa root nodule. The level of hybridization was 100 % in all combinations.

An antiserum raised against strain 042B could agglutinate strain 042B and the re-isolates from both soybean and

alfalfa root nodules. All cultures yielded the same agglutination value (1:1024).

These experiments showed that strain 042B could nodulate and fix nitrogen in association with soybean. Therefore, the possibility that our preliminary results were due to contamination by soybean rhizobia was ruled out.

Strain 042B and *B. japonicum* USDA110 were compared for symbiotic efficiency on soybean grown under hydroponic conditions for 38 d (Table 2). There were no marked differences in time of appearance of nodules, total nitrogenase activities, plant top dry weights and total nitrogen (Table 2). These results indicated that strains 042B and USDA110 were equally effective. The number of nodules produced by strain 042B was obviously greater than that of strain USDA110, but total nodule weight was similar in both strains. The strain 042B nodules were smaller (mean weight of single nodule, 5.5 mg) than strain USDA110 nodules (10.1 mg).

To examine nodulation and nitrogen fixation by strain 042B with soybean, unsterilized soil was used in soil pot experiments and strain USDA110 was employed as a standard. The aim of soil pot nodulation experiments was

Table 4. Soil pot experiment with strains 042B and USDA110 analysed in the harvest stage of soybean plants

The values are means of three replicates $\pm$ SE. Superscripts a and b indicate statistical similarity and dissimilarity in pairwise comparison.

Strain	Pod number	Seed wt (g)	Weight of 100 seeds (g)	Seed number	Seed nitrogen (%)
042B	78 ^a $\pm$ 8	27.57 ^a $\pm$ 2.36	19.83 ^a $\pm$ 0.64	151 ^a $\pm$ 10	6.38 ^a $\pm$ 0.26
USDA110	80 ^a $\pm$ 11	26.58 ^a $\pm$ 3.35	19.15 ^a $\pm$ 0.55	163 ^a $\pm$ 25	6.53 ^a $\pm$ 0.28
Uninoculated	63 ^b $\pm$ 13	21.37 ^b $\pm$ 2.59	18.14 ^a $\pm$ 1.20	121 ^b $\pm$ 19	5.85 ^b $\pm$ 0.31

Table 5. Nodule occupancy (%) of strains 042B and USDA110 in different growing stages of soybean plants in unsterilized soil

Growing stage	Strain	
	042B	USDA110
Flowering stage	85	80
Pod stage	90	86
Seed stage	82	78

to estimate the commercial value of strain 042B in the field. Table 3 shows that the total nitrogen content of soybean plants inoculated with strain 042B or USDA110 was somewhat higher during the growing season than that of uninoculated control. In the harvest season, the pod number, seed number, seed weight and total nitrogen of seeds from soybean plants inoculated with 042B or USDA110 were higher than those of the uninoculated control, but there were no differences between those with strains 042B and USDA110 (Table 4).

Competition between strain 042B and indigenous rhizobia tested by ELISA

Twenty soybean root nodules nodulated by indigenous rhizobia were picked out randomly for ELISA, and none reacted with the antisera raised against strains USDA110 and 042B.

To determine nodule occupancy at different times during the growing season, random samples of 80 nodules were taken from soybean plants inoculated with strain 042B or USDA110 and tested by ELISA. The results (Table 5), showing greater than 78% nodule occupancy, indicate that, under unsterilized soil pot conditions strains 042B and USDA110 both have strong competitive abilities.

Physiological, biochemical and taxonomic characteristics of strain 042B

Strain 042B grew faster than strain USDA110; under optimal conditions, the generation time of strains 042B

Fig. 1. Agarose gel electrophoresis of plasmid DNA. Lanes: A, Rm41; B, USDA110; C, 042B; D, USDA1002.

and USDA110 were 2.0 h and 9.6 h, respectively. Strain 042B tolerated higher salt concentration, growing in the presence of 5% NaCl, while strain USDA110 was inhibited by 0.5% NaCl. Strain 042B adapted to alkaline conditions (pH 10.7) and a wider range of temperature (10–42 °C).

In *R. meliloti* 41, three plasmids of 60, 200 and 1250 kb have been reported. Of these, the 60 kb plasmid is an exogenous plasmid RP4 with resistance to kanamycin, tetracycline and ampicillin (Yu *et al.*, 1992). We detected two large plasmids in strain 042B (Fig. 1). They appeared to be similar in size to those in *R. meliloti* USDA1002, which have been estimated to be 200 kb and > 1000 kb. Strain USDA110 appeared to have no plasmids under our culture conditions.

To confirm the taxonomic position of strain 042B, DNA G+C mol% and DNA homology of this strain and related fast- and slow-growing soybean rhizobia were determined. The G+C mol% of 63.6 of strain 042B is in the same range as other rhizobia, and those determined for other reference strains were in their published ranges (Jordan, 1984) (Table 6). DNA homology between strain 042B and other alfalfa rhizobia was more than 70% except for strain CC169, which belongs to division B of *R. meliloti* according to Eardly *et al.* (1992). DNA homology

Table 6. DNA homology (%) between strain 042B and reference strains

Species	Strain	G + C mol%	Homology with 042B (%)
<i>R. meliloti</i> group A	042B	63.6	100
	USDA1002 ^T	63.6	76
	102F28	63.2	82
	CC2013	63.3	71
<i>R. meliloti</i> group B	CC169	63.1	40
<i>R. fredii</i>	RT19	63.1	5
	USDA205 ^T	62.8	10
	CCBAU110	63.1	38
<i>B. japonicum</i>	USDA110	64.3	14
	USDA6 ^T	64.3	12

between strain 042B and soybean rhizobia was less than 40% (Table 6).

DISCUSSION

In this work, we have demonstrated that strain 042B, isolated from alfalfa in Xinjiang Autonomous Region of China, is able to nodulate and fix nitrogen in association with soybean plants. We have shown that this strain is, in other respects, a typical *R. meliloti*. However, none of the other *R. meliloti* strains that we tested was able to nodulate soybeans. Indeed, such an ability has never previously been reported for this species. The fact that strain 042B could infect and fix nitrogen in soybean plants indicates that the symbiotic capabilities of *R. meliloti* are not necessarily confined to alfalfa and closely related legumes, as was generally believed until now.

Since Xinjiang is in the north-west border area of China, with a typical continental climate, having hot summers and cold winters, the temperature there varies greatly between day and night. It is the driest area in China with high saline alkalinity. The *R. meliloti* 042B growing in this environment has characteristics consistent with such a habitat. Despite the symbiotic and physiological peculiarities of strain 042B, we found that it still belongs to the species *R. meliloti* based on DNA homology analysis with strain 042B and other rhizobia (Table 6). According to Eardly *et al.* (1990), alfalfa rhizobia can be divided into two divisions, A and B. Our results indicate that strain 042B belongs in division A.

Jarvis *et al.* (1992) indicated that the sequence of part of the gene encoding 16S rRNA of *R. meliloti* was identical to that of fast-growing soybean rhizobia, hence a close phylogenetic relationship has been established. In this case, partial sequence of 16S rRNA would not allow one to distinguish between *R. meliloti* and *R. fredii*. However, DNA homology between them is low. It is possible that they were closely related in the past before evolving into different species.

However, the most interesting question about strain 042B is the nature of its *nod* genes which may be distinctive. The *nod* regulatory genes are involved in the detection of plant signals secreted in root exudates, generally flavonoids. Plant flavonoids activate *nod* structural genes which, in turn, bring about the production of the Nod factors that activate the transcription of plant symbiotic genes. Nod factors secreted by *R. meliloti* are mono-*N*-acylated chitin oligomers that are *O*-acetylated and *N*-acylated at the non-reducing end and sulfated at the reducing end. Interestingly, *Bradyrhizobium japonicum* and *R. fredii* produce very similar Nod factors which are substituted with a methyl fucose group on the C6 of the reducing glucosamine residue (Denarie *et al.*, 1993). Hence, strain 042B might secrete a mixture of sulfated and non-sulfated Nod factors which signal back to alfalfa and soybean plants.

Strain 042B has a high symbiotic efficiency on soybeans, displaying early appearance of nodules and a higher nitrogenase activity relative to *B. japonicum*. Its associated plant total nitrogen and seed weight are similar to strain USDA110. In addition, strain 042B can grow under unfavourable conditions, grows faster and utilizes a wider range of carbohydrates than most strains that nodulate soybeans. These advantages are beneficial to the industrial production of rhizobia inoculum. Therefore, strain 042B has a commercial value, but it remains to be studied further in the field.

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REFERENCES

- Button, J. C. (1980). New developments in inoculating legumes. In *Recent Advances in Biological Nitrogen Fixation*, pp. 380–405. Edited by N. S. Subba Rao. London: Arnold Press.
- 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., Wu, B. H., Luo, C. H., Li, J. L., Shi, Y. H., Huang, L. & Wu, Z. Y. (1987). The survey and taxonomy of leguminous root nodule bacteria of Xinjiang region. *Sci Agric Sin* **20**, 22–27 (in Chinese).
- 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.
- Denarie, J., Debelle, F., Truchet, G. & Promé, J.-C. (1993). Rhizobium and legume nodulation: a molecular dialogue. In *New Horizons in Nitrogen Fixation*, pp. 19–30. Edited by R. Palacios *et al.* The Netherlands: Kluwer Academic Publishers.
- Eardly, B. D., Materon, L. A., Smith, N. H., Johnson, D. A., Rumbaugh, M. D. & Selander, R. K. (1990). Genetic structure of

natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. *Appl Environ Microbiol* **56**, 187–194.

Eardly, B. D., Young, J. P. W. & Selander, R. K. (1992). Phylogenetic position of *Rhizobium* sp. strain Or 191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *nifH* genes. *Appl Environ Microbiol* **58**, 1809–1815.

Eckhardt, T. (1978). A rapid method for the identification of plasmid DNA in bacteria. *Plasmid* **1**, 584–588.

Fuhrmann, J. & Wollum, A. G., II (1985). Simplified enzyme-linked immunosorbent assays for routine identification of *Rhizobium japonicum* antigens. *Appl Environ Microbiol* **49**, 1010–1013.

Hardy, R. W. F., Burns, R. C. & Holsten, R. D. (1973). Applications of the acetylene–ethylene assay for measurement of nitrogen fixation. *Soil Biol Biochem* **5**, 47–81.

Jarvis, B. D. W., Downer, H. L. & Young, J. P. W. (1992). Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*. *Int J Syst Bacteriol* **42**, 93–96.

Johnson, J. L. (1985). Determination of DNA base composition, DNA reassociation and DNA hybridization of bacterial nucleic acid. *Methods Microbiol* **18**, 1–74.

Jordan, D. C. (1984). Family III. Rhizobiaceae Conn 1938, 321^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. I, pp. 234–242. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Marmur, J. & Doty, P. (1962). Determination of the base composition of DNA from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.

Sadowsky, M. J., Bohlool, B. B. & Keyser, H. H. (1987). Serological relatedness of *Rhizobium fredii* to other rhizobia and to the bradyrhizobia. *Appl Environ Microbiol* **5**, 1785–1789.

Schmidt, E. L., Bankole, R. O. & Bohlool, B. B. (1968). Fluorescent antibody approach to the study of rhizobia in soil. *J Bacteriol* **95**, 1987–1992.

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

Vincent, J. M. (1982). *Nitrogen Fixation in Legumes*. Sydney: Academic Press.

Yang, S. S. & Li, J. L. (1988). Salt tolerance and nodulation characteristics of fast-growing soybean rhizobia. *Acta Agric Univ Pek* **14**, 143–148 (in Chinese).

Yu, Y., Xu, L. M., Fan, H. & Ge, C. (1992). A study on the plasmid profiles and plasmid deletion of *Sinorhizobium fredii* strains from different origins. *Sci Agric Sin* **25**, 14–21 (in Chinese).

Zhou, P. J., Den, J. L., Zhang, X. J. & Wu, S. T. (1979). Nodulation experiments with legume using a hydroponic method. *Oil Crops China* **2**, 60–62 (in Chinese).

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