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Bradyrhizobium liaoningense sp. nov., Isolated from the Root Nodules of Soybeans

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Seventeen strains of extra-slowly growing (ESG) soybean rhizobia isolated from root nodules of *Glycine soja* and *Glycine max* growing in five provinces (Liaoning, Heilongjiang, Shanxi, Hubei, and Anhui) in the People's Republic of China were compared with 48 reference strains belonging to the genera *Bradyrhizobium*, *Rhizobium*, and *Agrobacterium* by performing a numerical analysis of 191 phenotypic features. Our results showed that all of the ESG strains examined clustered closely in the genus *Bradyrhizobium* but were separated from *Bradyrhizobium japonicum* at the species level and that they could be differentiated from *Rhizobium* and *Agrobacterium* species at the genus level. On the basis of the results of our numerical taxonomy analysis, a genomic DNA G+C content analysis, DNA-DNA hybridization experiments, a partial 16S rRNA sequence analysis, a serological analysis, an N and C content analysis, and an N/C ratio analysis of members of the three groups of soybean rhizobia, we propose the name *Bradyrhizobium liaoningense* sp. nov. for the ESG strains; the type strain of this species is strain 2281.

Bradyrhizobium japonicum (formerly *Rhizobium japonicum*) was recognized as the sole symbiont of soybeans for a long time, until Keyser et al. (13) isolated fast-growing rhizobia from soil and soybean nodules collected in the People's Republic of China in 1982. In 1979 Gross et al. (5) described extra-slowly growing (ESG) soybean rhizobia, but their strains appeared to be *B. japonicum* strains. In 1982, we first isolated ESG strains from nodules of *Glycine soja* collected from different regions of Liaoning Province in the People's Republic of China and then isolated ESG strains from nodules of soybean cultivars grown in Shanxi, Heilongjiang, Anhui, and Hubei Provinces (25). So far, the following three groups of soybean symbionts have been identified: the fast-growing soybean rhizobia, the slowly growing soybean rhizobia, and the ESG soybean rhizobia. These groups differ significantly in their physiological and biochemical properties, generation times, serological characteristics (25, 26), G+C contents, levels of DNA homology (16), and partial 16S rRNA sequences.

The slowly growing soybean rhizobia were transferred from the genus *Rhizobium* to the single species of the genus *Bradyrhizobium*, *B. japonicum*, by Jordan (10, 11). Later, the new species *Bradyrhizobium elkanii* was described by Kuykendall et al. (14). The fast-growing soybean rhizobia were placed in one species of the genus *Rhizobium*, *Rhizobium fredii*, by Scholla and Elkan (19), and in 1988 Chen et al. described the new genus *Sinorhizobium*, which contained two species, *Sinorhizobium fredii* and *Sinorhizobium xinjiangensis* (1a). In 1992 Jarvis et al. performed a phylogenetic study in which they used partial 16S rRNA sequencing and concluded that classification of the fast-growing soybean rhizobia in a separate genus was not justified and that the name *R. fredii* should be retained (8).

In order to determine the taxonomic position of the ESG rhizobia, we studied ESG strains and reference strains by performing a numerical analysis, a serological typing analysis, a G+C content analysis, a DNA-DNA hybridization analysis, and a 16S rRNA sequence analysis. On the basis of the results of this study and previously published data from an N and C content analysis, we propose the name *Bradyrhizobium liaoningense* for the ESG strains.

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MATERIALS AND METHODS

Strains. The bacterial strains used in this study are listed in Table 1. The purity of the strains was ensured by using single-colony isolates, checking colony morphology on yeast extract-mannitol agar (YMA) (23) and yeast extract–L-arabinose agar, and examining Gram stain reactions.

Growth conditions. The ESG strains were maintained on yeast extract–L-arabinose agar, which contained 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.2 g of $CaSO_4$, 0.4 g of yeast extract powder, 10 g of L-arabinose, 20 g of agar, and enough distilled water to bring the volume to 1,000 ml (pH 6.5 to 6.8). *Azorhizobium caulinodans* ORS571^T (T = type strain) was maintained on sodium lactate medium, which contained 1.67 g of K_2HPO_4, 0.87 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of NaCl, 40 mg of $CaCl_2$, 4 mg of $FeCl_3$, 0.4 g of yeast extract powder, 10 g of sodium lactate, 20 g of agar, and enough distilled water to bring the volume to 1,000 ml (pH 6.8). All of the other strains were maintained on YMA (23).

Phenotypic features and analytical methods. A total of 65 strains were characterized by studying the following features: (i) utilization of L-rhamnose, D-arabinose, L-(+)-arabinose, L-fucose, D-(+)-raffinose, D-xylose, D-mannose, L-sorbose, fructose, D-galactose, D-cellobiose, inulin, D-(+)-melezitose, D-turanose, D-lyxose, D-trehalose, maltose, lactose, sucrose, glucose, D-(−)-ribose, D-melibiose, D-(−)-tagatose, mannitol, sorbitol, dulcitol, inositol, *meso*-erythritol, glycerol, isopropanol, adonitol, ethylene glycol, esculin, salicin, creatinine, L-glutamine, casein hydrolysate, sodium lactate, ammonium oxalate, sodium citrate, sodium D-gluconate, vanillic acid, calcium malonate, sodium succinate, sodium D-malate, sodium pyruvate, sodium hippurate, urea, ammonium tartrate, D-arabitol, L-glutamic acid, L-cysteine, glycine, salicyl alcohol, pyruvic acid, sodium benzoate, sodium alginate, fumaric acid, vanillin, ferulic acid, malic acid, *p*-aminobenzoic acid, sodium salicylate, tannic acid, and biuret (each at a concentration of 0.1%) as sole carbon sources; (ii) utilization of DL-arginine hydrochloride, L-methionine, 6-furfurylaminopurine, thymine, L-threonine, L-serine, DL-threonine, L-tryptophan, L-lysine, glycine, D-serine, DL-methionine, DL-phenylalanine, L-cysteine, DL-proline, L-arginine, D-methionine, L-cystine, L-tyrosine, L-leucine, L-proline, L-isoleucine, L-histidine, L-valine, DL-α-alanine, DL-valine, aspartic acid, D-histidine, arginine monohydrochloride, L-glutamine, asparagine, L-glutamic acid, and cytosine (each at a concentration of 0.1%) as sole nitrogen sources; (iii) requirement for inositol (0.25 mg/ml), biotin (0.025 mg/ml), vitamin B_{12} (0.025 mg/ml), vitamin B_5 (0.025 mg/ml), vitamin E (0.025 mg/ml), vitamin B_1 (0.025 mg/ml), pyridoxine hydrochloride (0.025 mg/ml), vitamin C (0.025 mg/ml), folic acid (0.025 mg/ml), and nicotinamide (0.025 mg/ml); (iv) tolerance to dyes, including nigrosine, neutral red, basic fuchsin, safranine T, malachite green, acridine orange, bromophenol blue, bromocresol green, Sudan I, and brilliant cresyl blue (each at a concentration of 0.1%); (v) tolerance to the antibiotics lincomycin (5 and 25 U/ml), ampicillin (5 and 25 μg/ml), gentamicin (5 and 25 U/ml), chlortetracycline (5 and 25 U/ml), bleomycin (5 and 25 μg/ml), oxytetracycline (5 and 25 μg/ml), carbenicillin (5 and 25 μg/ml), neomycin (10 and 30 U/ml), kanamycin (10 and 50 μg/ml), bacitracin (5 and 25 μg/ml), chloramphenicol (10 and 50 μg/ml), doxycycline (5 and 25 U/ml), oxacillin (5 and 25 U/ml), erythromycin (25 and 100 U/ml), penicillin (25 and 50 μg/ml), and tetracycline (25 and 50 U/ml); (vi) growth at pHs 5, 8, 9, and 10; (vii) growth on YMA supplemented with NaCl at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 M; (viii) reaction in litmus milk; (ix) growth in peptone broth; (x) reduction

TABLE 1. Origins and hosts of the bacterial strains studied

Strain(s)	Host(s)	Origin[a]	Source[b]
ESG soybean rhizobia			
2060, 2061, 2062, 2064, 2067, 2068, 2044, 2043	*G. soja*	Liaoning Province	SFI (CAAS) (L. M. Xu)
2312, 2279, 2309	*G. max*	Liaoning Province	SFI (CAAS) (L. M. Xu)
2080	*G. max*	Shanxi Province	SFI (CAAS) (L. M. Xu)
2281^T, 2260	*G. max*	Heilongjiang Province	SFI (CAAS) (L. M. Xu)
DE454, DE544	*G. max*	Hubei Province	SFI (CAAS) (M. L. Jiang and C. Ge)
2325	*G. max*	Anhui Province	SFI (CAAS) (L. M. Xu and H. Fan)
Bradyrhizobium japonicum strains			
2040, 2045	*G. soja*	Liaoning Province	SFI (CAAS) (L. M. Xu)
2242, 2261	*G. max*	Heilongjiang Province	SFI (CAAS) (L. M. Xu)
2088	*G. max*	Shanxi Province	SFI (CAAS) (L. M. Xu)
2228	*G. max*	Ningxia Province	SFI (CAAS) (L. M. Xu)
2319	*G. max*	Anhui Province	SFI (CAAS) (L. M. Xu and H. Fan)
2287	*G. max*	Jiangxi Province	SFI (CAAS) (L. M. Xu)
005	*G. max*	Shandong Province	SFI (CAAS)
002	*G. max*	Guizhou Province	SFI (CAAS)
C223	*G. max*	Henan Province	IOC (CAAS) and SFI (CAAS) (M. L. Jiang and C. Ge)
113-2	*G. max*	Hubei Province	IOC (CAAS)
ATCC 10324^T	*G. max*	Japan	USDA (from S. S. Young)
Bradyrhizobium sp. (*Lupinus*) strain G13		Argentina	SFI (CAAS)
Bradyrhizobium elkanii 61A76	*G. max*	United States	SFI (CAAS)
Rhizobium fredii strains			
2048, 2049, 2059	*G. soja*	Liaoning Province	SFI (CAAS) (L. M. Xu)
2075	*G. max*	Jiangsu Province	NAU
2078, 2092	*G. max*	Shanxi Province	SFI (CAAS) (L. M. Xu)
2201, 2205	*G. max*	Ningxia Province	SFI (CAAS) (L. M. Xu)
2241, 2307, 2300	*G. soja*, *G. max*	Heilongjiang Province	SFI (CAAS) (L. M. Xu)
2268	*G. max*	Xinjiang Province	SFI (CAAS) (L. M. Xu)
2245, 2251	*G. max*	Jiangxi Province	SFI (CAAS) (L. M. Xu)
2271A	*G. max*	Guangdong Province	SFI (CAAS) (L. M. Xu)
2265	*G. max*	Shandong Province	SFI (CAAS) (L. M. Xu)
2341, 2350	*G. max*	Hebei Province	SFI (CAAS) (L. M. Xu)
2336	*G. max*	Anhui Province	SFI (CAAS) (L. M. Xu and H. Fan)
C331	*G. max*	Henan Province	IOC (CAAS) and SFI (CAAS) (M. L. Jiang and C. Ge)
Gd 306	*G. max*	Hubei Province	HAU
DE145, DH532	*G. max*	Hubei Province	IOC (CAAS) and SFI (CAAS) (M. L. Jiang and C. Ge)
USDA257		Shanxi Province	USDA
USDA191		Shanghai Province	USDA
USDA205^T	*G. max*	Henan Province	USDA (from S. S. Young)
Rhizobium loti ATCC 33669^T	*Lotus corniculatus*		CCBAU
Rhizobium meliloti ATCC 9930^T	*Medicago sativa*	Virginia	CCBAU
Rhizobium huakuii CCBAU 2609^T	*Astragalus inicus*	Nanjing, People's Republic of China	NAU
Rhizobium leguminosarum bv. viciae ATCC 10004^T	*Pisum sativum*	United States	CCBAU
Rhizobium leguminosarum bv. trifolii 540-80		New Zealand	SFI (CAAS)
Rhizobium leguminosarum bv. phaseoli 127K17		United States	SFI (CAAS)
Rhizobium sp. (*Astragalus*) strain ACCC 13070	*Astragalus adsurgens*	Beijing, People's Republic of China	ACCC
Agrobacterium tumefaciens T37			IBAC
Azorhizobium caulinodans ORS571^T			SFI (CAAS)
Rhizobium tropici ATCC 49672^T		Mexico	SFI (CAAS)
Rhizobium galegae 540^T			CCBAU

[a] All provinces are provinces in the People's Republic of China.

[b] SFI (CAAS), Soils and Fertilizers Institute, Chinese Academy of Agricultural Sciences, Beijing, People's Republic of China; IOC (CAAS), Institute of Oil Crops, Chinese Academy of Agricultural Sciences, Wuhan, People's Republic of China; NAU, Nanjing Agricultural University, Nanjing, People's Republic of China; HAU, Huazhong Agricultural University, Wuhan, People's Republic of China; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, People's Republic of China; ACCC, Agricultural Culture Collection of China, Beijing, People's Republic of China; IBAC, Institute of Botany, Academica Sinica, Beijing, People's Republic of China; USDA, United States Department of Agriculture, Beltsville, Md.

of methyl blue; (xii) nitrate reduction; (xiii) growth at 4 to 8°C and 35 to 37°C; (xiv) production of urease, catalase, β-galactosidase, and penicillinase; (xv) acid or alkali production on YMA; and (xvi) colony morphology.

Most of the biochemical tests were performed on plates inoculated with a multipoint inoculator (3) by using about 10^5 cells per point as the inoculum. Other tests were conducted in broth or on slopes. Cultures were incubated at 28°C unless indicated otherwise. The results for the ESG, slowly growing, and fast-growing strains were scored after 10 to 14, 7 to 10, and 3 to 7 days, respectively. Utilization of carbohydrates and organic acids was determined on the medium of White (24) containing the trace elements of medium Cs7 (18). Utilization of compounds as sole nitrogen sources was examined by using the same medium, except that the $NaNO_3$ was replaced with various nitrogen sources. Requirements for vitamins and growth factors were studied by using the medium of Zhou and Cao (29). Tolerance to antibiotics (12), tolerance to NaCl

at various concentrations, and tolerance to dyes were determined on YMA. The ability to grow in acid and alkaline media was determined on YMA plates in which the pH was adjusted to 5.0, 8.0, 9.0, and 10.0. Growth in peptone broth was determined by the usual method. The reaction in litmus milk was determined as described previously (21), and the results were observed after 1 to 5 weeks of incubation. Nitrate reduction was tested by routine identification methods used for bacteria (21). Methyl blue reduction was studied by adding 1 drop of 1% methyl blue to a test tube containing inoculated milk and incubating the tube at 28°C for 7 to 14 days and then at 37°C for 1 h; disappearance of the blue color was considered a positive reaction. Generation times were determined by using the method of Yelton et al. (27). Urease, catalase, and β-galactosidase activities were detected by using the method of Smibert and Krieg (21); penicillinase activity was detected by using the method of Foley and Pervet (3). Colony morphology was evaluated on YMA.

Computer analysis. Characteristics were coded 1 for positive results and 0 for negative results. Similarity values were calculated by using the S_{sm} formula (22), the center strain of each phenotypic group was determined, clustering by average linkage was performed by the methods of Sneath and Sokal (22), and a dendrogram was produced by using the MINTS system of the Computer Research Laboratory at the Institute of Microbiology of Academica Sinica, Beijing, People's Republic of China.

Serological analysis. Serological typing of ESG strains was performed by using the method of Vincent (23).

Plant infection tests. ESG strains were examined for their symbiotic relationships with leguminous plants. Tests were performed in jars by using the method of Vincent (23). The nitrogenase activity of nodules was determined by using the ethylene reduction method (6, 15).

N and C contents of cell components. The N and C contents of cell components were determined by using the method of Sheng and Jin (20).

DNA base composition. DNAs were extracted by using the method of Johnson (9), and the G+C contents of the DNAs were determined by the thermal denaturation method (2, 9, 17).

Levels of DNA homology. Levels of DNA homology were determined by using the initial renaturation rate method (2, 9, 17).

Nucleotide sequences of partial 16S rRNA genes. A 260-bp segment of the DNA that encodes the 16S rRNA of ESG strain 2062, corresponding to positions 44 to 337 in the *Escherichia coli* sequence, was amplified by the PCR and was sequenced by using the method of Young et al. (28). Calculation of genetic distances and construction of the phylogenetic tree was done by the methods of Chen et al. (1).

Nucleotide sequence accession number. The ribosomal gene sequence of ESG strain 2062 has been deposited in the EMBL, GenBank, and DDBJ databases under accession number X86065.

RESULTS AND DISCUSSION

A total of 65 strains, including 17 ESG soybean rhizobial strains and 48 reference strains belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*, were compared by performing a numerical analysis of 191 phenotypic features (Fig. 1). All of the strains examined were linked at a similarity level of 62%, while the 17 ESG strains, 13 *B. japonicum* strains, and one *Bradyrhizobium* sp. (*Lupinus*) strain clustered at a similarity level of 75% (group I) and the fast-growing soybean rhizobia and other members of the genera *Rhizobium* and *Agrobacterium* grouped together at a similarity level of 70% (group II) (Fig. 1). Phenotypic groups I and II corresponded to the genera *Bradyrhizobium* and *Rhizobium*, respectively.

We recognized two phena within group I, which contained ESG, *B. japonicum*, and *Bradyrhizobium* sp. (*Lupinus*) strains. All of the ESG strains were closely linked at a similarity level of 85% and formed an independent phenon (phenon 1). The high level of similarity for this phenon showed that all of the ESG strains were closely related to each other. Phenon 2 included 14 *Bradyrhizobium* strains which clustered at a similarity level of 81.5%; this phenon was further divided into three subphena, which contained 1 strain (2261), 12 strains, and 1 strain (2045). We observed distinct patterns for the three subphena in the carbon and nitrogen utilization tests, the antibiotic resistance tests, and the tests used to determine the ability to produce acid and alkali on YMA. All of these subphena exhibited the diversity of *B. japonicum* (28).

Group II included 33 members of the genus *Rhizobium* (26 strains of fast-growing soybean rhizobia and 7 representative strains of other *Rhizobium* species) and one strain belonging to the genus *Agrobacterium*. The 34 strains in this group were divided into four phena. Twenty-one strains of fast-growing soybean rhizobia (*R. fredii*) clustered as one phenon (phenon 1) at a similarity level of 83%. Three strains that represented *Rhizobium leguminosarum* biovar viciae, *R. leguminosarum* biovar trifolii, and *R. leguminosarum* biovar phaseoli grouped together at a similarity level of 88% (phenon 2). *Rhizobium loti* and *Rhizobium huakuii* strains were linked at a similarity level of 85% and formed another phenon (phenon 3). The fourth phenon consisted of four strains of *R. fredii*, one strain of *Rhizobium meliloti*, and one strain of *Agrobacterium tumefaciens*, which clustered at a similarity level of 81%. Group II contained two strains, ACCC 13070 (isolated from *Astragalus* sp.) and 2049 (*R. fredii*), which did not fall into any of the four phena.

The differential phenotypic characteristics of phenon 1 of group I (ESG strains), phenon 2 of group I (*B. japonicum*), and group II (*Rhizobium* spp. and other reference strains) are shown in Table 2. The ESG strains were quite different from the *B. japonicum* strains in their carbon source utilization patterns. The colonies of the ESG strains were smaller than those of the *B. japonicum* strains and were punctiform on YMA. Morphologically, the bacteroids of ESG and *B. japonicum* were rod shaped, but Y-shaped bacteroids of ESG were also found during scanning electron microscopic observations (26). In addition, the extraordinarily long generation times of the ESG strains and their strong ability to produce alkali distinguished these organisms from *B. japonicum* strains (Table 3).

Most ESG strains (including strains not included in this study) could be placed in three somatic antigen serogroups (serogroups 2060, 2062, and 2068) (26), and the members of each serogroup did not cross-react with the members of the other serogroups; the only exceptions were strains 2279, 2260, and 2325, which did not fall into any of these three serogroups and may be representatives of new serogroups. Representative antigens from members of the three serogroups did not cross-react with antisera from members of 15 *B. japonicum* serogroups and five *R. fredii* serogroups, while only ESG strains 2260 and 2325 reacted weakly with serogroup 002 antiserum and ESG strain 2279 produced a moderate reaction with serogroup 113-2 antiserum (*B. japonicum*). Our results indicated that there was not much serological relationship between the ESG strains and *B. japonicum* or *R. fredii* except for strain 2279. Moreover, all of the ESG strains described by Gross et al. belong to serogroup 135 of *B. japonicum* (5).

There are major differences in the nitrogen and carbon contents of whole cells and the ratio of N to C among ESG, *Bradyrhizobium*, and *Rhizobium* strains, and these parameters can be used as taxonomic tools (4). In ESG strains, the N and C contents are 9.19 to 11.62 and 42.56 to 46.10%, respectively, and the ratio of N to C is 20.56 to 25.46. *B. japonicum* cells contain 5.41 to 9.71% N and 42.68 to 50.32% C, and the ratio of N to C is 11.43 to 20.94; in addition, the ratio of N to C for one strain of *B. elkanii* falls within this range. *R. fredii* cells contain 2.74 to 4.33% N and 50.82 to 52.73% C, and the ratio of N to C is 5.27 to 7.59. Our results indicate that the ESG strains differ significantly from *B. japonicum*, *B. elkanii*, and *R. fredii* in their nitrogen and carbon contents and in the ratio of N to C in their cell components.

Twelve ESG strains isolated from Liaoning Province were used to examine cross-infection in leguminous species. None of the strains nodulated *Pisum sativum*, *Lotus* sp., *Astragalus sinicus*, and *Melilotus* sp. (26). Several strains nodulated *Phaseolus aureus* poorly, and low levels of nitrogenase activity were observed (6, 15). However, the ESG strains nodulated *Glycine*

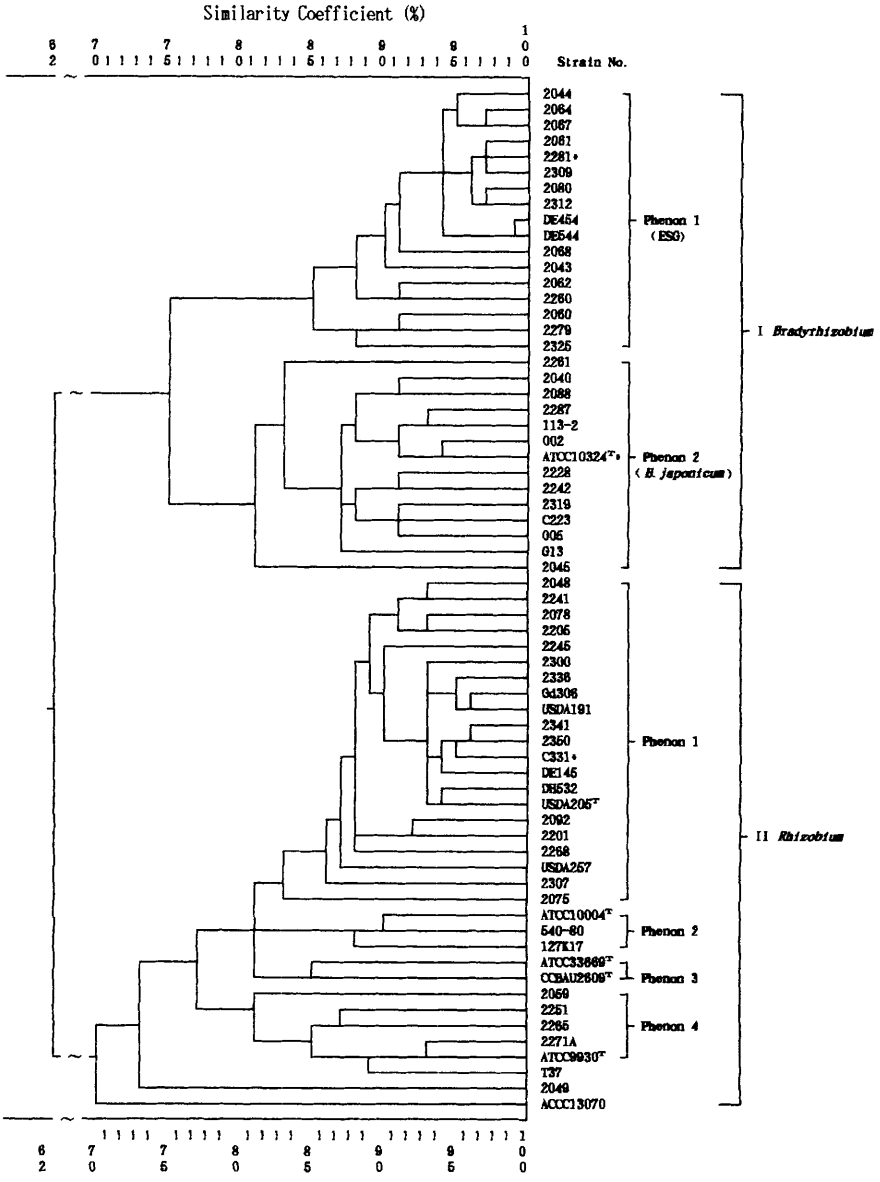

FIG. 1. Phenetic dendrogram showing the relationships among ESG strains, *B. japonicum* strains, *R. fredii* strains, and other reference strains. An asterisk indicates the central strain of each phenon.

max (cultivar Heinong 26) excellently with high nitrogenase activity, and the total amount of nitrogen that accumulated was comparable to the amounts accumulated by *B. japonicum* USDA110 and ATCC 10324^T.

The G+C contents of the ESG strains ranged from 60 to 64 mol% (16), values which fall within the range of values for the genus *Bradyrhizobium*.

DNA hybridization experiments were performed with five representative ESG strains belonging to different serogroups that had different geographical origins and *Bradyrhizobium*, *Rhizobium*, *Azorhizobium*, and *Agrobacterium* strains. The five ESG strains which we used exhibited high levels of relatedness to each other (range, 71.6 to 93.4%) low levels of relatedness to *B. japonicum* and *B. elkanii* (Table 4), and very low levels of relatedness to *Rhizobium* spp., *Azorhizobium caulinodans*, and *Agrobacterium tumefaciens* (3.8 to 21.5%). Taking into account our previously published data (16), we concluded that all of the ESG strains tested were closely related to each other and exhibited low levels of relatedness to *B. japonicum* (7), *B. elkanii* (14), and other reference strains.

The nucleotide sequence of the 16S rRNA of ESG strain 2062 was compared with the corresponding previously published sequences of *B. japonicum* USDA59, *B. elkanii* USDA76, *Bradyrhizobium* sp. strain BTAi1, *Rhodopseudomonas palustris* 216, *Azorhizobium caulinodans* ORS571^T, *R. fredii* USDA205^T, *Rhizobium galegae* LMG6214^T, *R. leguminosarum* bv. phaseoli 8002, *R. loti* NZP2213^T, and *Agrobacterium tumefaciens* DSM 30105, which differed at 5, 11, 1, 7, 33, 34, 38, 38, 40, and 45 nucleotide positions, respectively. However, the sequence of 2062 is identical to that of *B. japonicum* USDA110. The sequence data showed that the ESG strains were closely related to BTAi1, USDA59, 216, and USDA76 (Fig. 2).

On the basis of the results of our numerical taxonomy anal-

TABLE 2. Differential characteristics of ESG soybean rhizobia, *B. japonicum*, *Rhizobium* spp., and *Agrobacterium tumefaciens*

Characteristic	% of positive strains		
	ESG soybean rhizobia	*Bradyrhizobium japonicum*	*Rhizobium* spp. and *Agrobacterium tumefaciens*
Carbon source utilization			
D-(+)-Raffinose	0	20-79	90-99
Fructose	11-19	90-99	100
D-Galactose	11-19	90-99	100
D-Cellobiose	0	20-79	100
Inulin	0	20-79	100
D-(+)-Melezitose	0	20-79	80-89
D-Turanose	1-10	100	90-99
D-Trehalose	0	20-79	90-99
Maltose	0	90-99	100
Lactose	0	20-79	100
Sucrose	0	20-79	100
Glucose	11-19	100	100
D-Melibiose	0	20-79	90-99
Tagatose	11-19	20-79	20-79
Mannitol	1-10	90-99	100
Sorbitol	1-10	20-79	100
Inositol	0	1-10	90-99
meso-Erythritol	0	20-79	100
Glycerol	0	90-99	90-99
Adonitol	1-10	20-79	100
Ethylene glycol	0	20-79	20-79
Esculin	0	20-79	20-79
Salicin	0	0	90-99
Sodium citrate	1-10	20-79	80-89
Calcium malonate	11-19	20-79	90-99
Ammonium tartrate	0	90-99	20-79
D-Arabitol	11-19	100	100
Nitrogen source utilization			
6-Furfurylaminopurine	1-10	100	100
Thymine	0	20-79	100
Tolerance to:			
Chlortetracycline (25 μg)	100	100	1-10
Bleomycin (5 μg)	11-19	20-79	100
Oxytetracycline (25 μg)	100	100	1-10
Chloramphenicol (50 μg)	1-10	20-79	11-19
Doxycycline (25 U)	100	100	0
Penicillin (25 μg)	0	100	80-89
Penicillin (50 μg)	0	20-79	80-89
Tetracycline (50 U)	80-89	100	1-10
NaCl (0.1 M)	0	0	100
NaCl (0.2 M)	0	0	90-99
NaCl (0.3 M)	0	0	20-79
Methyl blue reaction	0	0	80-89
Growth in peptone broth	0	0	20-79
Growth at pH 10	0	0	90-99
Growth at:			
35°C	0	1-10	90-99
37°C	0	0	80-89
Reaction in litmus milk			
Peptonization	0	0	80-89
Reduction	20-79	20-79	100

ysis, serological typing analysis, N and C content analysis, DNA base composition analysis, DNA homology analysis, 16S rRNA sequence analysis, and cross-inoculation tests, we propose that the ESG strains isolated in the People's Republic of China should be classified in a new species of the genus *Bradyrhizobium*, *Bradyrhizobium liaoningense* (li.a.o.ning.en'se. M. L. adj., *liaoningense*, referring to Liaoning, a province in the People's Republic of China from which rhizobia were isolated).

Description of *Bradyrhizobium liaoningense* sp. nov. Rods are about 0.5 by 1.5 to 4.5 μm. Spores are not formed. Gram negative. The colonies are circular, entire, semitranslucent, raised, non-mucoid, and usually 0.2 to 1 mm in diameter within 7 to 14 days on YMA. The strains utilize a narrow range of carbohydrates, organic acids, and amino acids as sole carbon sources. All of the ESG strains tested utilize arabinose, glutamic acid, and sodium glutamate. Most strains assimilate rhamnose, xylose, mannose, xylose, ribose, glutamine, sodium lactate, sodium gluconate, sodium pyruvate, and sodium succinate. The ESG strains use cystine, isoleucine, histidine, valine, aspartic acid, arginine monohydrochloride, glutamine, glutamic acid, cytosine, and asparagine as sole nitrogen sources. Most strains also utilize methionine, serine, tryptophan, lysine, proline, arginine, tyrosine, leucine, and proline. The ESG strains have an extralong generation time. Alkali production occurs on YMA (pH up to 7.45 to 8.08) (Table 3). The optimum growth temperature range is 25 to 30°C, and the optimum pH is 6.5 to 6.8. The tolerance of the ESG strains to antibiotics is lower than that of other *Bradyrhizobium* spp. The ESG strains are susceptible to bleomycin (25 μg/ml), erythromycin (100 μg/ml), and penicillin (25 μg/ml), but are resistant to lincomycin (25 U/ml), neomycin (30 U/ml), bacitracin (25

TABLE 3. Generation times of ESG strains and alkali production by these strains in yeast extract-mannitol liquid medium

Strain	Generation time (h)	pH of medium[a]
2060	23.6	7.86
2044	29.4	7.77
2061	31.4	7.70
2062	29.2	7.78
2064	26.2	8.08
2067	31.6	7.60
2068	39.6	8.04
2080	31.3	7.88
2260	18.2	7.62
2281[T]	28.2	7.76
2279	24.4	7.64
2309	29.6	7.63
2312	36.6	7.68
DE454	34.2	7.66
DE544	20.5	7.90
2043	19.0	7.49
2325	16.4	7.45

[a] The pH values were determined after cultivation for 4 days; the initial pH was 6.8.

TABLE 4. Levels of DNA homology between representative ESG strains belonging to different serogroups from different geographical areas[a]

Strain	% DNA homology with:				
	Strain 2062	Strain 2068	Strain 2080	Strain 2309	Strain DE544
2062	100				
2068	93.4	100			
2080	90.9	92.3	100		
2309	71.6	73.2	86.6	100	
DE544	90.4	72.1	82.2	85.6	100
ATCC 10324 (DNA group I)	14.0	19.0	25.4	35	9.1
USDA110 (DNA group Ia)	14.0	31.0	30.0	38.4	36.7
61A76 (*B. elkanii*) (DNA group II)	23.0	7.3	25.8	29.6	14.0

[a] The DNA homology values are the means of the values from two or three replications. The calculated relative error was less than 5%.

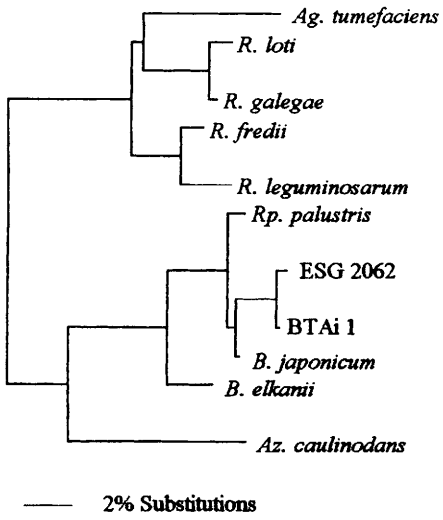

FIG. 2. Phylogenetic tree showing the positions of ESG strain 2062, *Rhodopseudomonas palustris* 216, and other taxa in the family *Rhizobiaceae*. *Az. caulinodans*, *Azorhizobium caulinodans*; *Ag. tumefaciens*, *Agrobacterium tumefaciens*; BTAi 1, *Bradyrhizobium* sp. strain BTAi1.

μg/ml), gentamicin (25 U/ml), chlortetracycline (25 U/ml), doxycycline (25 U/ml), oxacillin (25 U/ml), and oxytetracycline (25 μg/ml). Additional characteristics are shown in Table 2. The type strain of *B. liaoningense* is 2281, which is the center strain of this species group. This strain conforms to the description given above for the species.

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