Deoxyribonucleic Acid Homology Between Fast-Growing Soybean-Nodulating Bacteria and Other Rhizobia†

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Deoxyribonucleic acid homologies were determined between five fast-growing soybean-nodulating strains from the People's Republic of China and other rhizobia. Type strains *Rhizobium trifolii* ATCC 14480, *Rhizobium meliloti* ATCC 9930, *Rhizobium phaseoli* ATCC 14482, *Rhizobium leguminosarum* ATCC 10004, and *Bradyrhizobium japonicum* ATCC 10324 were used to represent recognized species. *Bradyrhizobium* sp. (*Lupinus*) strain ATCC 10319, the type strain of the former species *Rhizobium lupini*, and *Bradyrhizobium* sp. strain 3G4b5 were also included. The fast-growing soybean-nodulating strains had low levels of deoxyribonucleic acid homology with the type strains studied (5 to 43%). The fast-growing soybean-nodulating strains studied constitute at least two distinct deoxyribonucleic acid homology groups.

The root nodule bacteria which form symbioses with soybeans (*Glycine*) were previously classified as *Rhizobium japonicum*, but recently this group of organisms has been transferred to a new genus, *Bradyrhizobium* (5). *Bradyrhizobium japonicum* is a member of the slow-growing group of rhizobia that are characterized by generation times in excess of 6 h. Recently, fast-growing (generation times, less than 6 h) soybean-nodulating (FGSN) strains have been isolated from the People's Republic of China (7). Although these Chinese isolates are capable of nodulating several cultivars of *Glycine max*, effective symbiosis by all of the strains has only been reported with *Glycine max* cv. Peking and the wild soybean, *Glycine soja* (7). One strain, USDA 191, has been reported to nodulate American soybean cultivars effectively (16). However, the nitrogen-fixing capabilities of this symbiosis are relatively low (16). Since the FGSN strains have cultural characteristics of the fast-growing rhizobia (7, 14, 16) and symbiotic characteristics of a slow grower, *B. japonicum*, the relationship of these strains within the genera *Rhizobium* and *Bradyrhizobium* is unknown (5, 7).

In this study we examined the deoxyribonucleic acid (DNA) homology of five FGSN strains with the type strains of each species of *Rhizobium*. DNA homology within the FGSN group was also examined.

MATERIALS AND METHODS

Bacterial strains. FGSN strains USDA 193, USDA 194, USDA 201, USDA 205, and USDA 206 were supplied by Harold Keyser, United States Department of Agriculture, Beltsville, Md. Strains USDA 194, USDA 201, USDA 205, and USDA 206 were isolated from soybean nodules collected in Honan Province, China. Strain USDA 193 was isolated from nodules collected in Shansi Province (Harold Keyser, personal communication). The type strains of *Rhizobium meliloti*, *Rhizobium phaseoli*, *Rhizobium leguminosarum*, *Rhizobium trifolii*, and *B. japonicum*, as designated on the Approved Lists of Bacterial Names (15), were obtained from the American Type Culture Collection, Rockville, Md. Type strain ATCC 10319 of the former species *Rhizobium lupini* (6) was obtained from the American Type Culture Collec-

tion. Cowpea strain 3G4b5 was obtained from the North Carolina State University culture collection. The authenticity of the rhizobial cultures was ensured by plant testing on appropriate hosts. Strain 3G4b5 was plant tested on *Arachis*. FGSN strains were plant tested on *Glycine max* cv. Peking.

Media and culture conditions. *Rhizobium* cultures for inocula were grown on a rotary shaker (200 rpm) at 28°C in 50-ml Delong culture flasks containing 20 ml of yeast extract-mannitol broth (9). *Rhizobium* cultures for DNA isolations were grown in HEPES-Mes (HM) broth (1). The carbon sources used included fructose, mannitol, and glycerol to increase cell yield and decrease extracellular polysaccharide production. Type strains were grown in 15-liter carboys containing 12 liters of HM broth and 1.0 ml of antifoam A (Sigma Chemical Co., St. Louis, Mo.). These containers were inoculated with 0.5 to 1.0 liters of cultures grown for 5 days (fast growers) or 7 days (slow growers) on a rotary shaker at 28°C. Other *Rhizobium* strains were grown to stationary phase in 2.8-liter Fernbach flasks containing 1 liter of HM broth. A 10-ml inoculum was used. The purity of *Rhizobium* strains was determined by plating onto yeast extract-mannitol agar containing bromthymol blue (50 mg/liter) and onto tryptic soy agar (Difco Laboratories, Detroit, Mich.).

DNA isolation. The lysis and isolation procedure used was the technique of Marmur (10), as modified by Ligon et al. (8). Cells were collected by centrifugation, washed with 50 ml of saline-ethylenediaminetetraacetate (0.15 M NaCl, 0.05 M ethylenediaminetetraacetate, pH 8.0), recentrifuged, and diluted to a concentration of 1 g of cells per 25 ml of saline-ethylenediaminetetraacetate. The cells were lysed by adding 1 ml of a 20% sodium lauryl sulfate solution per 10 ml of cell suspension, followed by incubation at 60°C for 15 min. The lysate was then treated with 1 volume of water-saturated phenol by shaking for 30 min. The emulsion was centrifuged at 10,000 × g for 15 min to separate the two phases. The aqueous phase was removed, sodium perchlorate was added to a concentration of 1 M, and the mixture was shaken for 30 min with an equal volume of chloroform-isoamyl alcohol (24:1); this was followed by centrifugation. The chloroform-isoamyl alcohol treatment was then repeated. The aqueous phase was removed, and the DNA was precipitated with 2 volumes of cold 95% ethanol. The DNA was dissolved in distilled water, and then NaCl was added to a concentration of 1 M. The DNA precipitation was repeated twice. The DNA solution was made 0.1 M to NaCl, 0.05 M to ethylenediaminetetraacetate, and tris(hydroxymethyl)aminomethane

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buffer. Ribonuclease was added to a concentration of 200 µg/ml, and the preparation was incubated for 60 min 60°C. Sodium lauryl sulfate was added to a concentration of 1%, and this was followed by treatment with pronase (50 µg/ml). The DNA solution was treated once with phenol and twice with chloroform-isoamyl alcohol, as described above. The DNA was then precipitated three times with 2 volumes of ethylene glycol monoethyl ether. After the third precipitation, the DNA was dissolved in water, sodium acetate was added to a concentration of 0.6 M, and the DNA was diluted to a concentration of 100 µg/ml with 0.6 M sodium acetate; 2 volumes of glycerol was added to the DNA, which was fragmented with a VirTis homogenizer (model 60K; microset up) at 4000 rpm for 2 min. The optimum fragment molecular weight for this hybridization procedure is 4×10^6 . This procedure produces fragments ranging in molecular weight from 3×10^6 to 5×10^6 , as determined by agarose gel electrophoresis. The fragmented DNA was precipitated by adding 2 volumes of 95% ethanol and stored at -20°C overnight. The DNA was harvested by centrifugation at $16,000 \times g$ for 30 min at 4°C. The supernatant was poured off, and the bottle was rinsed with 50 ml of 95% ethanol. This was again left overnight at -20°C and then centrifuged. The DNA precipitate was allowed to dry at room temperature and was dissolved in 0.03 M phosphate buffer (pH 6.8). Further purification and removal of single-stranded DNA were accomplished by passage through hydroxylapatite columns (1 g of hydroxylapatite per 400 µg of DNA). The hydroxylapatite was rehydrated in 0.03 M phosphate buffer and poured into water-jacketed columns (diameter, 2.5 cm) at 60°C. The columns were washed with 5 volumes of 0.03 M phosphate buffer. The DNA was then loaded onto the column; this was followed by sequential washing with 3 volumes each of 0.03, 0.14, and 0.42 M phosphate buffer. The eluate from the 0.42 M phosphate buffer washes, which contained double-stranded DNA, was then dialyzed against 0.6 M sodium acetate with three changes of solution. The DNA was precipitated as described above with 2 volumes of ethanol and overnight refrigeration (-20°C), followed by centrifugation. The precipitated DNA was dissolved in $0.1 \times$ standard saline citrate (SSC; $1 \times$ SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) and adjusted to a concentration of 150 µg/ml. Purity was determined by the ratios of absorbance at 260 nm to absorbance at both 230 nm (polysaccharide) and 280 nm (protein). Ratios above 2.30 and 1.95, respectively, were acceptable.

DNA reassociation. A spectrophotometric technique was used to determine levels of DNA homology. This technique has been described previously by Norgard and Bartell (12) and was modified for use with rhizobia, as described by Ligon et al. (8). The DNA reassociations were performed with a Beckman model DUR monochromator equipped with a Gilford model 252 photometer, a Gilford quartz microcuvette assembly with a model 2527 thermoprogrammer, and a Gilford model 6046 analog multiplexer. Samples to be hybridized were prepared as follows: (i) blank, 0.5 ml of $0.1 \times$ SSC; (ii) strain A, 0.5 ml of strain A DNA solution; (iii) strain B, 0.5 ml of strain B DNA solution; (iv) mixture, 0.25 ml each of strain A and strain B DNA solutions. To each of these solutions, 0.5 ml of a $12 \times$ SSC-50% formamide solution was added to yield a final concentration of 75 µg of DNA per ml in $6 \times$ SSC-25% formamide. Each of the four quartz microcuvettes was loaded with 0.25 ml of one of the prepared solutions. These were then heated to 95°C and held for at least 3 min to dissociate the double-stranded DNA. The samples were then cooled rapidly and maintained at

70°C. The absorbance values of the samples at 270 nm were monitored with a Gilford model 6051 chart recorder. A dwell time of 3 s, full-scale absorbance of 0.9 U, and a chart speed of 30 cm/h were used.

The rate of reassociation was followed as the rate of decrease in absorbance of the samples during the first 4 to 5 min, when the decrease was linear. The rates for each sample were determined graphically as the slope of the renaturation plots. The percentage of DNA base homology between samples was calculated by using the following formula:

$$\text{Percent homology} = 100 \times \left[\frac{4 \times V_{\text{mix}} - (V_1 + V_2)}{2\sqrt{V_1 \times V_2}} \right]$$

where V_{mix} was the renaturation slope of the DNA mixture, V_1 was the renaturation slope of DNA sample A, and V_2 was the renaturation slope of DNA sample B.

Previously, Ligon et al. (8) compared hybridization data from the spectrophotometric and hydroxylapatite batch techniques. This comparison indicated that data obtained by using the spectrophotometric technique have a mean deviation of less than 2% from data obtained by using the hydroxylapatite batch method under stringent (80°C) conditions (8).

RESULTS

The percentages of DNA homology of the FGSN strains hybridized with the type strain of each *Rhizobium* species are shown in Table 1. The mean level of DNA homology of 20 hybridizations with the type strains of *R. trifolii*, *R. meliloti*, *R. phaseoli*, and *R. leguminosarum* was 19%. The mean level of homology of 15 hybridizations with the type strains of *B. japonicum* and the former *R. lupini*, as well as with strain 3G4b5 (cowpea group), was 21%. These results indicated that, as a group, the FGSN strains were not closely related to other rhizobia, either fast or slow growing.

The levels of DNA homology of the FGSN strains when they were hybridized among themselves are shown in Table 2. Two distinct homology groups were identified. The first group contained strains USDA 194 and USDA 201, which were closely related to each other; the second group contained strains USDA 193, USDA 205, and USDA 206, which were also closely related to each other. Strain USDA 193 was also moderately related to the first group.

DISCUSSION

Recently, on the basis of biochemical and physiological properties, Sadowsky et al. (14) suggested that the FGSN strains are related to the other fast-growing groups of rhizobia. However, the levels of DNA homology indicates that these strains are not closely related to any of the present *Rhizobium* or *Bradyrhizobium* species. Diversity within the group exists, since there are two distinct homology groups. The first contains strains USDA 194 and USDA 201, and the second contains strains USDA 193, USDA 205, and USDA 206. Neither of the homology groups can be identified with a specific geographical location.

Little is known about the FGSN isolates. Masterson et al. (11) investigated the plasmid profiles of these strains and found that the location of *nif* genes is homologous to the structural *nifD* and *nifH* genes in *Klebsiella pneumoniae*. Sadowsky et al. (14) and Stowers and Eaglesham (16) investigated the physiological and symbiotic characteristics of the FGSN rhizobia, and Yelton et al. (17) conducted

TABLE 1. Levels of DNA homology between FGSN strains and other rhizobia

Strain	% Homology with FGSN strain:					Mean % homology
	USDA 194	USDA 201	USDA 193	USDA 205	USDA 206	
<i>R. trifolii</i> ATCC 14480 ^{Ta}	21	9	23	18	6	15
<i>R. meliloti</i> ATCC 9930 ^T	25	10	16	14	20	17
<i>R. phaseoli</i> ATCC 14482 ^T	35	23	22	7	26	23
<i>R. leguminosarum</i> ATCC 10004 ^T	11	22	40	5	27	21
<i>R. lupini</i> ATCC 10319 ^T	22	9	35	10	29	21
<i>B. japonicum</i> ATCC 10324 ^T	43	31	33	8	14	26
<i>Bradyrhizobium</i> sp. strain 3G4b5	12	24	5	18	28	17
Mean	24	18	25	11	21	

^a T = type strain.

extensive studies of strain USDA 191. The results of these authors indicate that the FGSN rhizobia are physiologically similar to the other fast-growing rhizobia.

Homologous strains USDA 194 and USDA 201 produce less acid in culture than the other FGSN strains (7) and also reduce litmus (14). Both of these strains harbor plasmids (11). Strain USDA 201 has *nif* genes on a plasmid, whereas these genes are not plasmid borne in strain 194 (11). Strains USDA 194 and USDA 201 are resistant to kanamycin (20 µg/ml), in contrast to strains USDA 193, USDA 205, and USDA 206 (16).

Studies of the symbiotic characteristics of the FGSN strains have indicated that they are as effective on soybean varieties Peking (7), Geduld, and Usutu (4) as slow-growing strains. Geduld and Usutu are varieties of South African origin which were derived from a genetic line imported from China (4). Megaplasmids may also play a role in the infectiveness of the FGSN strains (13). A comparison of the homology groupings described above with reports quantifying effectiveness does not indicate any major differences between the two groups. In a previous report from this laboratory, three homology groups were identified in *B. japonicum* (3). Results reported here indicate that the FGSN strains are not closely related to homology group I based upon hybridizations with *B. japonicum* strain ATCC 10324. Yelton et al. (17) have reported that FGSN strain USDA 191 is distantly related to strain *B. japonicum* USDA 110, a member of homology group Ia (3). Cowpea strain 3G4b5 is not closely related to the FGSN strains. In a study of 122 fast-growing *Rhizobium* strains, Crow et al. reported four homology groups (2). The results presented above indicate that the FGSN strains are not closely related to either group 1 (*R. leguminosarum*, *R. phaseoli*, and *R. trifolii*) or group 3 (*R. meliloti*), based upon hybridizations conducted with the type strains.

The FGSN strains do not appear to be closely related to the slow-growing rhizobia, based upon DNA homology and physiological characteristics. However, these strains are

symbiotically similar to the slow-growing soybean-nodulating strains. Keyser et al. (7) suggested that the FGSN rhizobia may be an intermediate group which links the fast- and slow-growing rhizobia. Our results indicate that the FGSN strains are a distinct group.

The taxonomic position of the FGSN bacteria is presently unresolved. Recent changes in the taxonomy of the root nodule bacteria have emphasized the physiological differences between the fast- and slow-growing groups (5). The FGSN bacteria cannot be classified as *B. japonicum*, because the genus *Bradyrhizobium* is reserved for slow-growing rhizobia, nor can they be classified as members of any present fast-growing species due to their symbiotic characteristics. As a result, proposals to assign an appropriate taxonomic designation should be entertained.

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TABLE 2. Levels of DNA homology within the FGSN group

Strain	% Homology with strain:				
	USDA 194	USDA 201	USDA 193	USDA 205	USDA 206
USDA 194	100	95	53	ND ^a	ND
USDA 201		100	63	24	41
USDA 193			100	92	96
USDA 205				100	91
USDA 206					100

^a ND, Not determined.

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