Rhizobium fredii sp. nov., a Fast-Growing Species That Effectively Nodulates Soybeans

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A new species, *Rhizobium fredii*, is proposed for fast-growing root nodule bacteria isolated from soybeans. The type strain was isolated from a root nodule of *Glycine max* growing in Honan Province, China, and is designated strain USDA 205 (= ATCC 35423 = PRC 205). This new species is differentiated from currently recognized *Rhizobium* and *Bradyrhizobium* species by deoxyribonucleic acid hybridization comparisons, plant specificity, generation times, antibiotic resistance, and serology. The strains of *R. fredii* are differentiated into two proposed chemovars, *R. fredii* chemovar *fredii* chemovar nov. and *R. fredii* chemovar *siensis* chemovar nov., by deoxyribonucleic acid hybridization tests, growth in the presence of erythromycin (20 µg/ml), final pH of yeast extract-mannitol medium, and serology.

Bacteria which form effective nitrogen-fixing nodules on the roots of leguminous plants are presently classified into two genera, *Rhizobium* and *Bradyrhizobium* (6, 8). Differentiation of these genera is based upon the cross-inoculation group concept (6, 8). The distinguishing features between the two genera are differences in physiology when the organisms are grown on medium containing yeast extract and mannitol and the plant host groups which are nodulated. These differences have been reviewed elsewhere (3, 4, 6, 8). *Rhizobium* spp. are considered to be fast growing, with generation times of less than 6 h, whereas *Bradyrhizobium* strains have generation times in excess of 6 h (6, 8). The genus *Rhizobium* presently includes three species, *Rhizobium meliloti*, *Rhizobium loti* (5), and *Rhizobium leguminosarum* (8), which includes the former species *Rhizobium trifolii* and *Rhizobium phaseoli* (7). *Bradyrhizobium* presently has one species, *Bradyrhizobium japonicum*, and includes strains that are capable of effectively nodulating lupines and soybeans (6). Also included within the bradyrhizobia is the cowpea miscellany (6).

Recently, a new group of fast-growing rhizobia which nodulate soybeans was isolated from mainland China (9). A list of these strains and appropriate references are shown in Table 1. Comparative studies of growth rate and acid production on yeast extract-mannitol medium (YEM) (9, 13, 15), plant cross-inoculation (9, 15), plasmid profiles (10, 12), location of nitrogen fixation (*nif*) genes (10), location of nodulation genes (12), susceptibility to antibiotics (15), physiological characteristics (9, 13, 15), vitamin requirements (15), tolerance to NaCl (13, 15), differentiation on litmus milk (13), and deoxyribonucleic acid (DNA) hybridization (14) indicate that this group has cultural characteristics of species in the fast-growing genus *Rhizobium* and symbiotic characteristics of species in the slow-growing genus *Bradyrhizobium*. DNA homology indicates that these fast-growing, soybean-nodulating strains are not genetically related to the type strain of any of the previously recognized species, but the strains in each proposed chemovar are related among themselves (14). Several investigators have indicated that this group of bacteria does not conform to the criteria for any of the previously recognized species (9, 10, 12–16). Therefore, we describe a new species of fast-growing rhizobia which nodulate soybeans.

The importance of the cross-inoculation concept as a taxonomic tool has recently been deemphasized (5, 6) because the results of several investigations have indicated that the traits of nodulation and nitrogen fixation are plasmid mediated (1, 2, 10–12). The importance of physiological differences is presently emphasized and is the basis for the recent reclassification of the slow-growing rhizobia into the genus *Bradyrhizobium* (6, 7). Since the fast-growing soybean-nodulating strains are physiologically similar to other fast-growing root nodule bacteria, we believe that the new strains are appropriately assigned to the genus *Rhizobium*.

The selection of a species name for root nodule bacteria traditionally has indicated the plant host group (5, 6, 8). Therefore, the fast-growing soybean strains might be named *Rhizobium japonicum*. This name was used previously for *Bradyrhizobium japonicum* (6). The confusion created by using the same name to describe two different groups of organisms is readily evident. Therefore, we propose that the new species be named in honor of the late Edwin Broun Fred (1887–1981). E. B. Fred pioneered *Rhizobium* research in the United States while serving as bacteriologist at Virginia Polytechnic Institute and State University from 1907 to 1913. In 1913, he joined the faculty of the University of Wisconsin, where he continued his many contributions to this field.

Rhizobium fredii sp. nov. *Rhizobium fredii* (fred'i.i. M.L. gen. n. *fredii* of E. B. Fred) cells are gram negative, non-sporeforming, and rod shaped. Motility is exhibited by only a small proportion (1 to 5%) of the strains; this proportion is increased when the organisms are cultured on soft agar. Cells from late log phase to stationary phase YEM cultures tend to become large and pleomorphic.

Within 7 days, colonies on YEM agar plates containing bromthymol blue are between 1.0 and 5.0 mm in diameter, produce an acidic reaction, and are circular, convex, and entire. Extracellular polysaccharide production is minimal, but some strains produce watery colonies. The amount of growth at 28°C on YEM agar containing 2% NaCl is variable, but all strains are inhibited at a concentration of 3%. Inhibition of all strains is evident at pH 9.5, but they grow well at pH 4.5.

Gelatinase, galactosidase, and 6-phosphogluconate dehydrogenase are produced. All strains grow on arabinose, cellobiose, dulcitol, fructose, fumarate, galactose, gluconate, glucose, glycerol, lactose, malate, mannose, pyruvate, raffinose, rhamnose, succinate, sucrose, trehalose, and xy-

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TABLE 1. Fast-growing soybean-nodulating rhizobia included in *R. fredii*

Strain	Reference(s)
USDA 191 (= PRC 440).....	9, 10, 12, 13, 16
USDA 192 (= OB2)	9, 10, 12-15
USDA 193 (= OB3)	9, 10, 12, -15
USDA 194 (= PRC 194).....	9, 10, 12-15
USDA 201 (= PRC 201).....	9, 10, 12-15
USDA 205 ^T (= PRC 205 ^T = ATCC 35423 ^T)	9, 10, 12-15
USDA 206 (= PRC 206).....	9, 10, 12-14
USDA 208 (= PRC 208).....	9, 12, 13, 15
USDA 214 (= PRC 214).....	9, 12, 13, 15
USDA 217 (= PRC 217).....	9, 12, 13, 15
USDA 257.....	13

lose. Litmus milk reactions are variable for acid and alkali, but most strains exhibit peptonization and reduction of litmus.

All strains are susceptible to gentamicin (30 µg/ml), rifampin (5 µg/ml), neomycin (100 µg/ml), and streptomycin (10 µg/ml). All strains are resistant to penicillin G (50 µg/ml). Resistance to kanamycin is variable at a concentration of 20 µg/ml, but all strains are susceptible to 50 µg of kanamycin per ml.

Large plasmids have been demonstrated in all strains. Some of the plasmids contain DNA regions that hybridize with probes for the *nifD* and *nifH* genes from *Klebsiella pneumoniae*. Plasmids involved in nodulation have also been identified. The levels of DNA-DNA homology with type strains *R. meliloti* ATCC 9930, *R. trifolii* ATCC 14480, *R. leguminosarum* ATCC 10004, *R. phaseoli* ATCC 14482, and *B. japonicum* ATCC 10324 range from 5 to 43%.

Effective nodules are formed on *Glycine max* cv. Peking (soybean), *Glycine soja* (wild soybean), *Vigna unguiculata* (cowpea), and *Cajanus cajan* (pigeon pea). An ineffective symbiosis is formed on *Macroptilium atropurpureum* (siratro), *Macroptilium lathyroides* (phasey bean), *Sesbania cannabina*, *Vigna radiata* (mung bean), and United States cultivars of *Glycine max* (soybean). *Leucaena leucocephala* (Haole Koa), *Medicago sativa* (alfalfa), *Trifolium repens* (white clover), *Trifolium pratense* (red clover), *Astragalus sinicus* (milkvetch), and *Arachis hypogaea* (peanut) are not nodulated.

The type strain of *R. fredii* is strain USDA 205, a culture of which has been deposited in the American Type Culture Collection as strain ATCC 35423. This strain conforms to the above description of the species. The levels of DNA hybridization with *R. meliloti* ATCC 9930^T (T = type strain), *R. trifolii* ATCC 14480^T, *R. phaseoli* ATCC 14482^T, *R. leguminosarum* ATCC 10004^T, and *B. japonicum* ATCC 10324^T are 14, 18, 7, 5, and 8%, respectively.

Within *R. fredii*, there appear to be two distinct subgroups which can be differentiated on the basis of DNA-DNA hybridization, acid production on YEM broth, kanamycin resistance, and serology. As a result, we propose two new chemovars, *Rhizobium fredii* chemovar *fredii* chemovar nov. and *Rhizobium fredii* chemovar *siensis* chemovar nov. Additional strains for which hybridization data are unavailable but serotyping has been done indicate that other serogroups exist (B. Bohlool, personal communication) and may warrant additional chemovar designations. These strains should be considered members of *R. fredii* chemovar unknown until more data are available.

R. fredii chemovar *fredii* is in accordance with the descrip-

tion of the species. The strains of this chemovar are susceptible to kanamycin (20 µg/ml), do not reduce litmus, and produce a final pH of less than 6.1 in YEM broth after 4 days of incubation at 28°C. The levels of DNA-DNA homology with strains USDA 205^T (= ATCC 35423^T) and USDA 201 are 91 to 96 and 24 to 63%, respectively. Included in this chemovar are strains USDA 193 and USDA 206 and type strain USDA 205.

R. fredii chemovar *siensis* is in accordance with the above description of the species and can be differentiated on the basis of kanamycin resistance (20 µg/ml), reduction of litmus, and a final pH in YEM broth of more than 6.1. The levels of DNA-DNA homology with strains USDA 201 and USDA 205^T (= ATCC 35423^T) are 95 and 24 to 53%, respectively. Included in this chemovar are strain USDA 194 and the type strain of the chemovar, strain USDA 201.

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