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Proposed Minimal Standards for the Description of New Genera and Species of Root- and Stem-Nodulating Bacteria

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Since the first volume of *Bergey's Manual of Systematic Bacteriology* was published, in 1984, two additional genera and several new species of stem- and root-nodulating bacteria have been proposed; further changes to the taxonomy of this group of organisms appear likely. This paper briefly reviews the current status of "Rhizobium" taxonomy and proposes minimal standards for the description of future genera and species belonging to this group of organisms.

The taxonomy of the root- and stem-nodulating bacteria of legumes is in a state of transition. The classification of these organisms based on plant infection (3, 21) has been abandoned after extensive criticism (23, 78) and the demonstration that the genes coding for nodulation, host specificity, and nitrogen fixation in fast-growing rhizobia are located on transmissible symbiotic plasmids (6, 48, 53). Introduction of Adansonian and holistic approaches (10, 12, 23) led initially to a consolidation of species but more recently has resulted in the description of additional genera and species. In some cases, this has led to confusion as to the proper terminology for these organisms and how they should be distinguished.

One of the functions of the subcommittees of the International Committee of Systematic Bacteriology is to recommend minimal standards for the valid publication of new taxa (38) and so to avoid the situation in which the literature includes many inadequately described bacteria for which no type strain is available. In this paper, the members of the International Subcommittee for the Taxonomy of *Rhizobium* and *Agrobacterium* briefly review recent developments in the taxonomy of root- and stem-nodulating bacteria and then propose minimal standards for their description.

Current taxonomy of the root-nodule bacteria of legumes. After reviewing data on the numerical taxonomy, DNA mole percent G+C values, nucleic acid hybridizations, cistron similarities, serological relationships, extracellular polysaccharide composition, carbohydrate utilization patterns, and metabolic capacities, antibiotic sensitivities, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein-banding patterns, and speed of growth of rhizobia on laboratory media, Jordan (31, 32) separated the root-nodule bacteria into two genera, *Rhizobium* and *Bradyrhizobium*. Those organisms previously designated *R. leguminosarum*. *R. trifolii*, and *R. phaseoli* were combined into a single species, *R. leguminosarum*, with three biovar designations. Two other species, *R. meliloti* and *R. loti*, were listed in this genus, but slowly growing nodule bacteria were transferred to the genus *Bradyrhizobium*, with the designation of a single

species, *B. japonicum*. This classification was clearly an interim one in that (i) it was based on rhizobia collected from only 15% of the 19,700 species of Leguminosae (1) and so was unlikely to be representative, (ii) it failed to consider the recently identified fast-growing rhizobia from soybean (34), and (iii) it grouped many slowly growing strains, including those from *Lotus* and *Lupinus*, as bradyrhizobia, without assigning them specific status.

Since 1984, two additional genera (*Azorhizobium* [15] and *Sinorhizobium* [9]) and five additional species (*R. galegae* [39], *Sinorhizobium fredii* [9, 59], *S. xinjiangensis* [9], *R. tropici* [46], and *A. caulinodans* [15]) of root- and stem-nodule bacteria have been proposed. Additional, currently unnamed, subgroupings have been identified in *R. meliloti* (19) and among rhizobia nodulating tree legumes (84). Stem-nodule symbionts of the genus *Aeschynomene*, which produce bacteriochlorophyll and appear to harvest light energy (17, 36), have also been identified but "fall unambiguously in the center of the *Bradyrhizobium* cluster" (82).

Strains of stem-nodulating bacteria isolated from *Sesbania rostrata* were examined by phenotypic and phylogenetic methods and shown to constitute a narrow cluster which could be represented as a single species (15). When rRNA:DNA hybridization showed a closer relationship of these organisms with *Xanthobacter* than with *Rhizobium* and *Agrobacterium*, a separate genus, *Azorhizobium*, was established, with one species, *A. caulinodans*. Organisms in this group are unusual in that they actively fix atmospheric nitrogen both symbiotically and ex planta (20).

The fast-growing rhizobia that nodulate soybean were first designated as *R. fredii* chemovars *fredii* and *siensis* (59). Subsequent studies with additional isolates from China led to the proposal that these organisms be placed in a separate genus, *Sinorhizobium*, comprising two species, *S. fredii* and *S. xinjiangensis* (9). It is generally agreed that these organisms constitute a unique group, warranting specific status (9, 59, 75), but classification of these organisms into a separate genus fails to consider the phenotypic (55) and genotypic (28, 29, 60, 75) relationships between fast-growing soybean rhizobia and *Rhizobium meliloti*. These organisms appear to be distinct from fast-growing *Acacia*, *Prosopis*, and *Leu-*

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caena rhizobia (84), but their relationship with fast-growing isolates from *Astragalus* remains to be determined.

Lindstrom (39) created the species *R. galegae* for *Rhizobium* strains isolated from *Galega orientalis*, justifying their specific status on the basis of their low DNA homology with other rhizobia; on bacteriophage typing, lipopolysaccharide, and protein banding patterns; and on metabolic differences (39–42, 75). Kajjalainen and Lindstrom (33) have also reported an analysis of restriction fragment length polymorphism among *Galega* strains.

Martinez et al. (45) distinguished two distinct groups among strains of *R. leguminosarum* bv. *phaseoli*. These differed in *nif* gene reiteration, host range, and ability to produce melanin (45, 46); in the electrophoretic mobilities of 15 metabolic enzymes (52); and in pH tolerance (24). Multilocus enzyme electrophoresis carried out with 65 type II strains (46) revealed further subdivision, with the type II strains now proposed as a single species, *R. tropici*, having two similar but distinct subgroups. Results with bean rhizobia parallel those obtained with rhizobia from tree legumes; Zhang et al. (84) reported eight clusters among isolates predominantly derived from *Leucaena*, *Acacia*, and *Prosopis*.

Phylogenetic approaches to the classification of root- and stem-nodule bacteria have paralleled those using more traditional methods. The hybridization of 16S rRNA to total DNA (14), the establishment of 16S rRNA nucleotide sequences (37), and most recently, the polymerase chain reaction (82) have all been used to analyze rRNA or the genes that encode it. Although data from these sources are considered to provide the best basis currently available for determining phylogenetic relationships among all bacteria (47), they have sometimes yielded relationships which differ from those determined on the basis of phenotypic differences. Phylogenetic studies place the rhizobia in the alpha subdivision of the Proteobacteria and group *R. meliloti*, *R. leguminosarum*, and *Agrobacterium tumefaciens* on a branch which includes *Rochalimaea quintana* and *Brucella*. *Azorhizobium caulinodans* and *Bradyrhizobium* spp. appear to be more closely related to *Rhodopseudomonas palustris* (27, 81, 82). Young (81) suggests that a revision of *Bradyrhizobium* and *Rhodopseudomonas* could even lead to the reclassification of the soybean symbionts as species of *Rhodopseudomonas*.

As the rhizobia are studied further and additional isolations are made, changes in their taxonomy will undoubtedly occur. The recent recovery from soil and from the rhizosphere of nonnodulating gram-negative rod-shaped bacteria having homology with *Rhizobium* (30, 61, 63) is a further taxonomic problem. Though all such isolates from the rhizosphere of bean clustered with *R. leguminosarum* bv. *phaseoli* (61) and are presumably noninfective strains from beans rather than ancestral forms of *Rhizobium*, they cannot currently be considered to be *Rhizobium*. Eight nonsymbiotic isolates tested by Segovia et al. (61) all nodulated *Phaseolus vulgaris* after the introduction of the symbiotic plasmid from CFN 42; 33% of the isolates examined by Jarvis et al. (30) acquired the ability to nodulate white clover following the introduction of the *F. leguminosarum* bv. *trifolii* symbiotic plasmid. Genomic reorganization in *R. leguminosarum* bv. *phaseoli* can lead to partial deletion of the symbiotic plasmid (64, 65), with nonsymbiotic forms in the rhizosphere of beans outnumbering those capable of nodulation by more than 40:1 (61).

Proposed criteria for establishing new genera and species of root- and stem-nodulating bacteria. It is essential that papers

proposing further change in the taxonomy of this group of organisms do so on the basis of both phylogenetic and phenotypic (symbiotic, cultural, morphological, and physiological) traits and following studies with a relatively large number of strains.

Bacterial nomenclature should reflect genomic relationships to the greatest degree possible (47, 58, 74). Schleifer and Stackebrandt (58) noted that "modern bacterial systematists should not be content to work with two types of classification: an artificial one for practical purposes—and a natural one with no practical applications." These authors list as reliable methods for the classification of procaryotic organisms techniques such as DNA-DNA hybridization, DNA-rRNA hybridization, the oligonucleotide sequence of 16S rRNA, and protein or multilocus enzyme electrophoresis but acknowledge that these traits alone are not sufficient for the classification of gram-negative bacteria. The Subcommittee on the Taxonomy of *Rhizobium* and *Agrobacterium* is in agreement with a phylogenetic focus but wants to ensure that those persons most likely to encounter unusual symbionts (field microbiologists and botanists) do not feel excluded from participation in taxonomic studies and have access to collaborators willing to undertake phylogenetic evaluations. Newly erected species must also be supported by phenotypic differences which can be used in strain identification. We foresee validly published species arising from a series of publications, in which initial phenotypic characterization with a balance of chromosomally determined and plasmid-mediated traits gives way to phylogenetic studies using techniques which may be available in only a few laboratories. Models for this type of approach include the work of Lindstrom and others with *R. galegae* (39–42) and that of Martinez and coworkers with the bean rhizobia (44–46, 52). The publication of a new genus or species would then be the culmination of considerable research rather than something lightly attempted.

Minimal standards for the description of new genera and species of root- and stem-nodule bacteria should include both study sample and descriptor requirements.

For *Xanthomonas campestris*, a species with taxonomic problems similar to those of *Rhizobium*, Vauterin et al. (69) concluded that an improved taxonomy would depend on examination of large, independently isolated sets of strains. For the rhizobia, where inoculation has been a major factor in the spread of particular strains, isolates should be chosen from different geographic regions but particularly emphasize the center or centers of origin of the host legume. Type strains and strains representative of genera and species that might be related to the test organisms must also be included. Traits to be considered in assigning specific status should include the following: symbiotic performance with selected hosts, cultural and morphological characteristics, DNA:DNA relatedness, rRNA:DNA hybridization and 16S rRNA analysis, DNA restriction fragment length polymorphisms, and multilocus enzyme electrophoresis.

Symbiotic performance with selected hosts. The ability of the organisms to form nodules with a range of leguminous hosts is still an important practical characteristic for the rhizobia and should be detailed. Host plants tested should include those currently recognized as hosts for the established species of rhizobia: *Medicago sativa*, *Pisum sativum*, *Phaseolus vulgaris*, *Trifolium repens*, *Lotus corniculatus*, *Glycine max*, *Vigna unguiculata*, *Leucaena leucocephala*, *Macropitium atropurpureum*, and *Galega officinalis* (7, 32), plus those hosts known to be nodulated by the strains being tested. The host plant from which each strain was isolated

should also be reported. Such plant tests have traditionally been done in Leonard jars (72), but growth pouches (Vaughn Seed Company, Downer's Grove, Ill.) could also be used. With growth pouch-grown plants and by using the root-tip-marking procedure of Bhuvaneswari et al. (4), differences in host range, nodulation efficiency, and the compatibility of host and rhizobia can be shown.

Cultural and morphological characteristics. Cultural and morphological characteristics which have been used in the taxonomy of the rhizobia include growth rate and colony characteristics on yeast extract-mannitol-mineral salts medium, presence or absence of an NADP-linked 6-phosphogluconate dehydrogenase (EC 1.1.1.43), and the ability to utilize glucose, sucrose, lactose, fructose, arabinose, succinate, or adipate as the sole carbon source for growth.

Strains of *Rhizobium* recognized to date have generation times of 2 to 4 h in yeast extract-mannitol-mineral salts medium and produce colonies that are usually 2 to 4 mm in diameter after 3 to 5 days of incubation. Growth rates for these organisms have usually been marginally faster than that reported for *A. caulinodans* (15). By contrast, most *Bradyrhizobium* strains have a generation time of 6 to 10 h and produce colonies that do not exceed 1 mm in diameter after 5 to 7 days of growth. Colony variation has been reported, however, in both *R. meliloti* (26) and *Bradyrhizobium* (18, 68).

Rhizobia isolated to date differ significantly in carbohydrate metabolism and substrate utilization. Fast-growing *Rhizobium* strains possess NADP-linked 6-phosphogluconate dehydrogenase activity and metabolize a wider range of carbohydrates than do *Azorhizobium* strains (15, 46, 84). By contrast, bradyrhizobia lack NADP-linked 6-phosphogluconate dehydrogenase activity but do metabolize a number of aromatic compounds (51). Ability to utilize glucose, sucrose, lactose, fructose, arabinose, succinate, and adipate could serve as a diagnostic test in the differentiation of currently recognized species of *Rhizobium*.

Serological methods and the analysis of cell lipopolysaccharide or protein-banding patterns following SDS-PAGE can also be used to characterize the rhizobia. Vincent and Humphrey (73) identified taxonomically significant group antigens; agglutination reactions (22, 70, 71), immunodiffusion in gels (16), immunofluorescence (5), and enzyme-linked immunosorbent assays in trays (35) or as dot blots (2, 50) have all been used to define particular rhizobia. Methods for the characterization of cell lipopolysaccharides following SDS-PAGE are given in detail by de Maagd et al. (13), Cava et al. (8), and Lipsanen and Lindstrom (42). de Maagd et al. (13) found few differences in the lipopolysaccharide-banding patterns of strains of *R. meliloti* but noted that the lipopolysaccharide of these organisms was distinctly different from those of both *R. leguminosarum* and *Agrobacterium*. Lipsanen and Lindstrom (42) also found the lipopolysaccharide and protein-banding patterns of *Galega* isolates to be different from those of other *Rhizobium* species.

Sufficient cultural, morphological, and other properties should be examined to permit the clustering of strains by numerical taxonomic methods. O'Brien and Colwell (49) list additional characterization tests, but new cultural and morphological tests which could be used in the routine identification of the different groups of rhizobia are always needed. Sackin (54) describes a number of programs which can be used for the numerical analysis of bacteria.

DNA-DNA relatedness. The DNA:DNA relatedness of strains representative of the proposed new species(s) should be determined by using established procedures (11, 27, 43,

75) and with reference to representatives of the known species of root- and stem-nodule bacteria, as well as those organisms not currently assigned specific status (i.e., the rhizobia from *Acacia* and *Astragalus*). The phylogenetic definition of a species would include all isolates having 70% or greater DNA:DNA relatedness and having ΔT_m values of 5°C or less (74). Species of *Rhizobium* usually have G+C values in the range 59 to 64 mol% (9, 32), while in *Azorhizobium* the range is 66 to 68 mol% G+C (15). *Bradyrhizobium* strains are intermediate between these groups, with a G+C value of 61 to 65 mol%.

rRNA:DNA and 16S rRNA analysis. Woese (79) considers rRNA to be the most useful of the highly conserved sequences available for the measurement of phylogenetic relationships. 16S rRNA analysis or rRNA:DNA hybridization should be applied to strains representative of newly identified groups of root- or stem-nodule bacteria. Methods for such analyses are detailed by De Smedt and De Ley (14), Lane et al. (37), and Young et al. (82). Data from sequence analysis can be compared with those from other *Rhizobium* species by using internationally available data bases, and if differences exist, dendrograms can be used to depict relationships. It should be recognized that different computer programs can yield different tree topographies. The use of programs which take care of differences in evolutionary rates has been recommended (47).

DNA restriction fragment length polymorphism. Chromosomal or plasmid DNA prepared from rhizobia, digested with restriction endonucleases, and subjected to electrophoresis in agarose gels (57) can be blotted onto nitrocellulose or nylon membranes (66) and hybridized with specific DNA probes. This procedure has been used extensively in *Rhizobium* but mainly to study *nod* and *nif* gene hybridization patterns (45, 56, 67). As currently employed, this methodology is likely to reinforce nodulation differences (33). Even so, its use was instrumental in the distinction of Type I and Type II strains from beans (45) and in identifying two distinct subgroups of *B. japonicum* (67). Recently, Wheatcroft and Watson (76, 77) identified the insertion sequence *ISRm1* in *R. meliloti* and showed that it was present in approximately 80% of the strains of this species. Additional DNA probes derived from other single-copy or reiterated chromosomal DNA sequences and other strains are needed.

Multilocus enzyme electrophoresis. Multilocus enzyme electrophoresis is a useful method for the initial characterization of potentially different groups of soil bacteria, including rhizobia (19, 25, 52, 80, 83). Since many of the genes for the enzymes evaluated are chromosomally located, multilocus enzyme electrophoresis data balance the emphasis traditionally given to plasmid-encoded symbiotic properties. Current methods for multilocus enzyme studies have been reviewed by Selander et al. (62). Enzymes used in studies with rhizobia have included the following: phosphoglucose isomerase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, leucylalanine peptidase, leucine aminopeptidase, hexokinase, adenylate kinase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, hydroxybutyrate dehydrogenase, aconitase, NAD-malate dehydrogenase, indophenol oxidase, and glutamate dehydrogenase (19, 46, 52).

Conclusions. In this paper, we have discussed those criteria which should be used in defining and assigning specific status to groups of root- and stem-nodule bacteria. Some represent traits controlled by a single gene and may be plasmid encoded; others explore phylogenetic relationships in DNA or RNA. Clearly, the greater the percentage of the

bacterial genome considered in the classification of strains, the greater the precision obtained.

This committee considers that a proper description of a new genus or species of root- or stem-nodule bacteria should be built on a set of independently isolated organisms and include both a numerical analysis of colonial and cultural characteristics and the analysis of representative strains by using measures of DNA:DNA relatedness and 16S rRNA analysis. Diagnostic tests which will enable the field worker to identify such organisms with reasonable precision should be noted in the description. The data needed for the effective classification of nonnodulating gram-negative, rod-shaped organisms having homology with *Rhizobium* would differ little from those outlined above, although the ability to nodulate following symbiotic plasmid transfer would be an important extra requirement. These requirements should not prevent scientists from publishing reports on new and interesting rhizobia—in fact, such publication is likely to promote further interest and collaborative study. They do aim to limit the premature naming of “new” species or genera, which cannot be retracted.

Finally, it is recommended that the *International Journal of Systematic Bacteriology* be the journal of choice for the valid publication of new or changed species designations, with all the type strains from such descriptions being deposited in an internationally recognized culture collection where they will be available to other workers.

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