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Taxonomy of rhizobia

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Abstract – Rhizobia are the bacteria that form nitrogen-fixing symbioses with legumes. Based on their characterisation by polyphasic taxonomy, their classification has undergone great changes in recent years. The current six rhizobium genera and 28 recognised species are reviewed here.

Rhizobium / taxonomy

Résumé – Taxonomie des rhizobia. Les rhizobia sont les bactéries qui forment des symbioses fixatrices d'azote avec des plantes de la famille des légumineuses. Suite à l'adoption de la taxonomie polyphasique comme critère de caractérisation, leur classification a subi de nombreux remaniements ces dernières années. Une revue de cette classification en six genres et 28 espèces actuellement reconnus est présentée ici.

Rhizobium / taxonomie

1. INTRODUCTION

Nitrogen fixation, that is to say the reduction of atmospheric nitrogen to ammonia, is the basic phenomenon making nitrogen available for the biosphere. Among living organisms, only some prokaryotes have this ability. They are spread over many of the major bacterial *phyla*. Among these, some soil bacteria, commonly and collectively called rhizobium or rhizobia, can form symbioses with leguminous plants. They induce the formation of specialised organs, called nodules, where they fix nitrogen to the benefit of the plant. These symbioses have major environmental and agricultural importance since they are responsible for most of the biological atmospheric nitrogen fixation on earth.

Rhizobia are diverse and, during recent years, their classification has undergone great changes due to new phylogenetic and polyphasic data leading to the description of new taxa [73]. Still it is an expanding field because more and more rhizobia are isolated and characterised every day, especially from the tropical and the mediterranean zones, where diversity is still poorly documented.

Until the early 1980s all symbiotic nitrogen-fixing bacteria from leguminous plants were classified in the single genus *Rhizobium*, including six species *R. leguminosarum*, *R. meliloti*, *R. trifolii*, *R. phaseoli*, *R. lupini* and *R. japonicum*. This taxonomy matched the cross-inoculation groups, defined as the set of host-plants nodulated by the same bacterial strains. For a long time

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the genera *Rhizobium*, *Agrobacterium*, and *Phyllobacterium* were grouped in the family *Rhizobiaceae* mainly based on their common functional characteristic of inducing cell proliferations on plants.

Taxonomy based on the concept of cross-inoculation groups failed because of the many exceptions to this rule. Moreover, at the same time, it was also widely recognized that *Rhizobium* classification should adjust to general bacterial taxonomy, and include a panel of genomic, phenotypic and phylogenetic features instead of the sole nodulation properties. Molecular techniques have developed considerably during the past 20 years and were made available for characterisation of rhizobia, leading to considerable changes in their classification.

2. POLYPHASIC TAXONOMY

Taxonomy studies the natural relationships existing between organisms and leads to their classification, a pre-requisite for the identification of any organism. Taxonomy [23] comprises (i) characterisation of the organisms, (ii) classification on the basis of similarity, (iii) nomenclature to give names to groups and (iv) identification of unknown organisms, to determine if they belong or not to one of the units classified and/or named. The central concept of bacterial taxonomy is the bacterial species. Unlike animals and plants, bacteria have simple morphological and physiological characteristics, limited and insufficient for taxonomic descriptions [68]. It is now recognised that bacterial classification must reflect the phylogenetic relationships between bacteria, main witnesses of which are the sequences of ribosomal RNA. In 1987, a group of taxonomists proposed a phylogenetic definition of the bacterial species based on various methodologies including the study of 16S ribosomal RNA and DNA/DNA hybridisations, but still recommending laying out discriminating phenotypic characteristics to name a new species [62]. Previously Colwell [9] founded the basis of modern bacterial taxonomy by recommending the integration of all pieces of information obtained at various levels of the cell - DNA, RNA, phenotypic and expressed features (proteins and their functions, fatty acids, chemotaxonomic markers) and by various techniques contributing to a polyphasic taxonomy likely to lead to a more stable classification. During the last twenty years, many techniques of bacterial characterisation have been developed at each level of information, and more computer facilities are becoming every day available for numerical analysis of data.

Each technique used in taxonomy has (i) its own discriminating power varying from the individual or species levels to the genus, family and higher levels, and (ii) its

field of application, dependent on the addressed question, the particular conditions, the number and the type of strains. The level of discrimination of a technique may vary depending on the studied bacterial taxon. In a polyphasic approach various complementary techniques with different discriminating levels are chosen to characterise the strains to be classified. The conclusion should be a consensus with minimal contradictions, drawing nomenclatural consequences and emphasising fast and reliable features for identification (preferably monophasic). Vandamme et al. [57] last reformulated the phylogenetic concept of the bacterial species as the assemblage of isolates which originated from a common ancestor population in which a steady generation of genetic diversity resulted in clones with different degrees of recombination and characterised by a certain degree of phenotypic consistency, by a significant degree of DNA/DNA hybridisation and by over 97% 16S rDNA sequence homology.

3. CURRENT RHIZOBIUM TAXONOMY

Rhizobia are rod bacteria, Gram-negative, aerobic, not sporulated [29]. Phylogenetically, they belong to the alpha subdivision of *Proteobacteria*. When it was recognised that the host spectrum was not the sole criterium to take into account for rhizobium classification, the *Rhizobium* species were first reclassified into two genera, the revised *Rhizobium* genus including the fast growing strains, and the new genus *Bradyrhizobium*, created for the slow growing ones [28]. Since then, isolation of rhizobia from an increasing number of plant species around the world and their characterisation by modern polyphasic taxonomy [57] has led to the description of additional new genera and species (Tab. I). Based on 16S ribosomal DNA sequences, the symbionts of leguminous plants belong to three main separate phylogenetic branches (Fig. 1).

3.1. *Rhizobium/Agrobacterium* branch

A first branch consists of several subbranches, each corresponding to four rhizobial genera, i.e. *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Allorhizobium*, intermixed with other genera, some plant-related bacteria (*Agrobacterium*, *Phyllobacterium*), soil bacteria (*Mycoplana*), and clinical bacteria (*Brucella*, *Ochrobactrum*, *Bartonella*). The first subbranch corresponds to the s.s. *Rhizobium* genus and includes *R. leguminosarum* (type species), *R. tropici* [37], *R. etli* [50], *R. gallicum* [2], *R. mongolense* [56] and *Agrobacterium* biovar 2. The second subbranch corresponds to the

Table I. Classification of nitrogen fixing bacteria forming symbioses with legume plants.

Species	Host-plant	References
Rhizobium		22
<i>R. leguminosarum</i>		22, 29
biovar viciae	<i>Pisum sativum</i> , <i>Vicia</i> , <i>Lathyrus</i> , <i>Lens</i>	22, 29
biovar trifolii	<i>Trifolium pratense</i>	22, 29
biovar phaseoli	<i>Phaseolus vulgaris</i>	22, 29
<i>R. tropici</i>		
Type II A	<i>Phaseolus vulgaris</i> , <i>Leucaena leucocephala</i>	37
Type II B	<i>Phaseolus vulgaris</i> , <i>Leucaena leucocephala</i>	37
<i>R. etli</i>		50
biovar phaseoli	<i>Phaseolus vulgaris</i>	24, 50
biovar mimosae	<i>Mimosa affinis</i> , <i>Leucaena leucocephala</i> , <i>Phaseolus vulgaris</i> L.	60
<i>R. hainanense</i>	<i>Desmodium sinuatum</i> & other plants of arid regions	8
<i>R. gallicum</i>		2
biovar gallicum	<i>Phaseolus vulgaris</i>	2
biovar phaseoli	<i>Phaseolus vulgaris</i>	2
<i>R. mongolense</i>	<i>Medicago ruthenica</i>	56
<i>R. galegae</i>		34
biovar orientalis	<i>Galega orientalis</i>	42
biovar officinalis	<i>Galega officinalis</i>	42
<i>R. giardinii</i>		2
biovar giardinii	<i>Phaseolus vulgaris</i>	2
biovar phaseoli	<i>Phaseolus vulgaris</i>	2
<i>R. huautlense</i>	<i>Sesbania herbacea</i>	59
Mesorhizobium		
<i>M. loti</i>	<i>Lotus corniculatus</i>	26
<i>M. huakuii</i>	<i>Astragalus sinicus</i> , <i>Acacia</i>	6
<i>M. ciceri</i>	<i>Cicer arietinum</i>	44
<i>M. tianshanense</i>	<i>Glycyrrhiza pallidiflora</i> & other tropical plants	7
<i>M. mediterraneum</i>	<i>Cicer arietinum</i>	45
<i>M. plurifarium</i>	<i>Acacia</i> , <i>Prosopis</i>	12
<i>M. amorphae</i>	<i>Amorpha fruticosa</i>	61
Sinorhizobium		5, 13
<i>S. meliloti</i>	<i>Medicago</i> , <i>Melilotus</i> , <i>Trigonella</i>	10, 13, 29
<i>S. fredii</i>		13, 49
chemovar fredii	<i>Glycine max</i>	49
chemovar siensis	<i>Glycine max</i>	49
<i>S. sahelense</i>	<i>Sesbania</i> spp.	13
biovar acaciae	<i>Acacia</i> spp.	4
biovar sesbaniae	<i>Sesbania</i> spp.	4
<i>S. terangae</i>		13, 55
biovar acaciae	<i>Acacia</i> spp.	36
biovar sesbaniae	<i>Sesbania</i> spp.	36
<i>S. medicae</i>	<i>Medicago</i>	48
<i>S. kostiense</i>	<i>Acacia</i> , <i>Prosopis</i>	43
<i>S. arboris</i>	<i>Acacia</i> , <i>Prosopis</i>	43
Azorhizobium		16
<i>A. caulinodans</i>	<i>Sesbania rostrata</i>	16
<i>Azorhizobium</i> sp.	<i>Sesbania rostrata</i>	47
Bradyrhizobium		28
<i>B. japonicum</i>	<i>Glycine max</i> , <i>Glycine soja</i>	29, 30
<i>B. elkanii</i>	<i>Glycine max</i>	31
<i>B. liaoningense</i>	<i>Glycine max</i> , <i>Glycine soja</i>	70
<i>Bradyrhizobium</i> sp.	<i>Vigna</i> , <i>Lupinus</i> , <i>Mimosa</i>	28
	<i>Acacia</i>	18
	<i>Aeschynomene</i>	1, 74
Allorhizobium		11
<i>A. undicola</i>	<i>Neptunia natans</i>	11

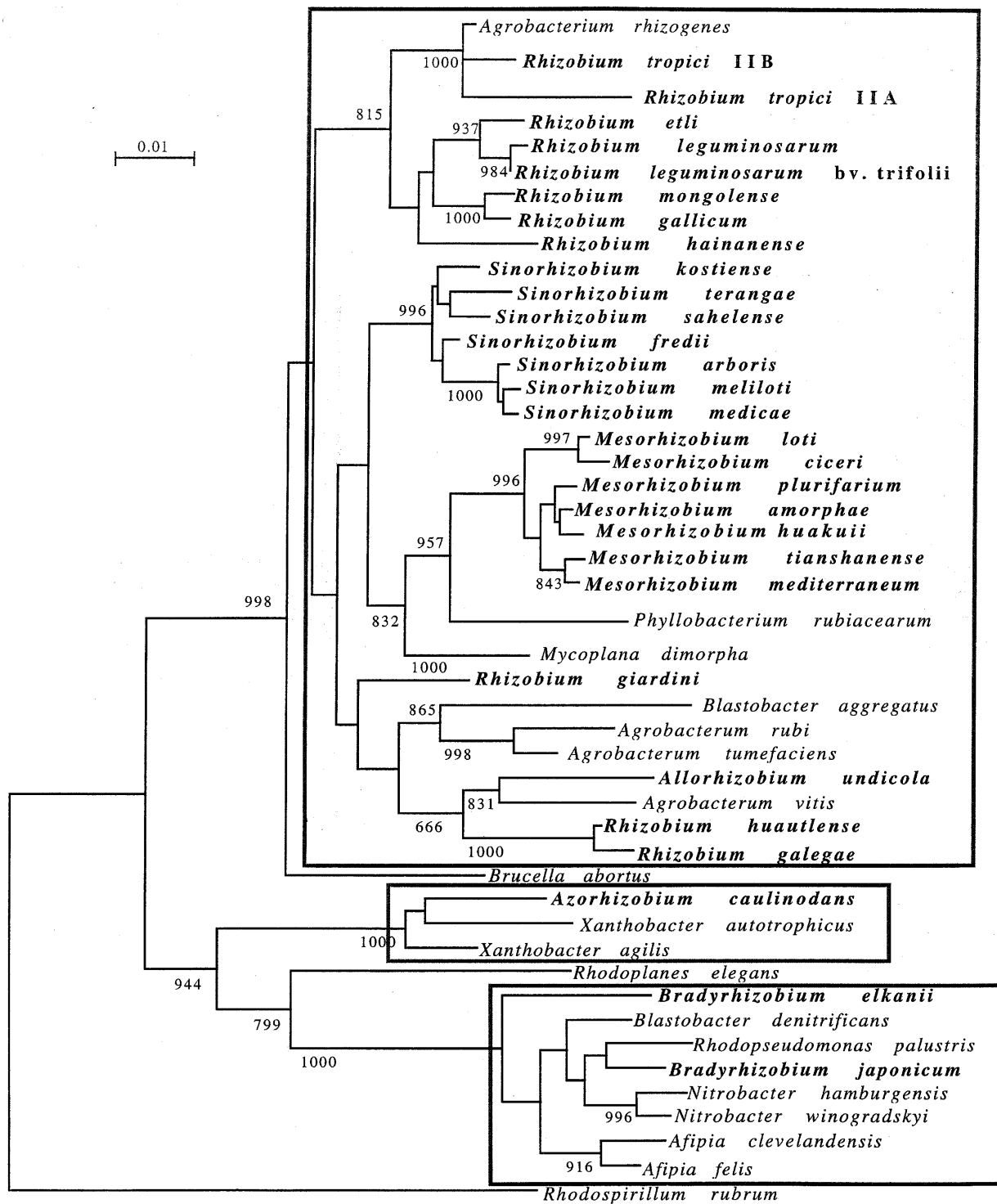


Figure 1. 16S rRNA gene sequence-based phylogenetic relationships among legume-nodulating rhizobial species (indicated in bold). Sequences were aligned using Clustal X software (Blosom matrix). The tree was constructed using the neighbour-joining method (PAUP4.0 software [52]) and rooted on *Rhodospirillum rubrum*. Significant bootstrap probability values, expressed as a percentage of 1000 replications, are given at the branching points.

Sinorhizobium genus and includes the species *S. fredii* and *S. xinjiangensis* [5], *S. meliloti*, *S. teranga* and *S. sahelense* [13, 55], *S. medicae* [48], *S. kostiense* and *S. arboris* [43]. The third subbranch corresponds to the genus *Mesorhizobium* [27] which contains *M. loti* [26], *M. huakuii* [6], *M. ciceri* [44], *M. mediterraneum* [45], *M. tianshanense* [7], *M. plurifarium* [12], *M. amorphae* [61]. The fourth subbranch includes *Agrobacterium* biovar 1, *Agrobacterium rubi*, *Agrobacterium vitis*, but also *R. galegae* [34], *R. giardinii* [2], *R. huautlense* [59] and *Allorhizobium undicola* [11]. Recently Young et al. [72] revisited taxonomic data from the literature. These authors argued that discriminatory phenotypic features to distinguish between these genera were not convincing, and that phylogenetic relationships between genera inferred from comparative 16S rDNA sequence analysis differ depending on the chosen algorithm and most particularly on the selection of included sequences. As a consequence, the authors proposed to group the three genera *Rhizobium*, *Agrobacterium* and *Allorhizobium* in a single emended genus *Rhizobium*, including all species of *Agrobacterium* and *Allorhizobium* as new combinations: *R. radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis* [72].

3.2. *Azorhizobium* branch

The second branch consists of *Azorhizobium caulinodans*, created for the rhizobia isolated from stem nodules of the tropical legume *Sesbania rostrata* [16]. These bacteria appeared to exhibit a number of very special features among rhizobia, like in vitro nitrogen fixation and assimilation for growth under low O₂ partial tension (3%). A second genomic species has been described in *Azorhizobium*, but not named [47]. Phylogenetically, the species of the genera *Azorhizobium*, *Xanthobacter* and *Aquabacter* are so intermixed that their inclusion in a single genus *Xanthobacter* has been examined but not proposed because of their many divergent phenotypic features [46].

3.3. *Bradyrhizobium* branch

The *Bradyrhizobium* genus was described by Jordan [28] to include all the so-called "slow growing" rhizobia, and for a long time, it comprised only one species, *B. japonicum*, including all soybean nodulating strains. However, Hollis et al. [25] had already shown that this group was heterogeneous and represented three DNA:DNA homology groups (I, Ia and II). For the group II, which differ from the species *B. japonicum* by many features, Kuykendall et al. [31] created a new

species, *Bradyrhizobium elkanii*. Other strains, with very slow growth (generation time between 16 and 24 hours), isolated from the nodules of *Glycine max* and *Glycine soya* in China, were given the proposed name *Bradyrhizobium liaoningense* [70].

B. japonicum [29] remains heterogeneous and contains at least two DNA/DNA hybridisation groups, I and Ia, whose representatives are respectively NZP 5549^T and USDA 110 [25]. The *Bradyrhizobium* genus is phylogenetically closer to non-symbiotic bacteria, like *Rhodopseudomonas palustris*, *Afipia*, *Nitrobacter* and *Blastobacter denitrificans*, than to *Rhizobium* and *Agrobacterium* [39, 63, 71]. Moreover, the integrity of the *Bradyrhizobium* genus is questionable since the type strain of *B. japonicum* is phylogenetically closer to *Afipia*, *R. palustris* and *Nitrobacter* than to *B. elkanii* and *Bradyrhizobium* sp. from Senegal and Brazil [3] (Fig. 1). The sequence of the gene coding for 16S rRNA of *B. liaoningense* is very close to that of *B. japonicum* [65]. Thus the taxonomy of the *Bradyrhizobium* genus remains confused. During the last few years, many groups of *Bradyrhizobium* sp. have been identified, and characterised by various phenotypic and genotypic methods, however drawing no taxonomic conclusions concerning their obvious positions in the classification [1, 3, 17, 18, 28, 32, 39, 40, 41, 51, 54, 58, 74], suggesting that the taxonomy of the *Bradyrhizobium* genus is still at a preliminary stage. Polyphasic taxonomy in *Bradyrhizobium* met with the problem that the different phenotypic and phylogenetic data did not lead to a clear consensus [18, 32, 38, 74].

Of special interest, photosynthetic *Bradyrhizobium* sp. strains were described, like strain BTAi1 [19], isolated from the stem nodules of *Aeschynomene indica*. It was shown to synthesise a pigment similar to the bacteriochlorophyll a [20, 21]. Since then, several authors have studied and described photosynthetic strains isolated from other species of *Aeschynomene* [32, 33, 35, 51, 69]. All photosynthetic strains form a monophyletic group on the *Bradyrhizobium* branch, separated from *B. japonicum* and *B. elkanii* [39, 69, 74].

In recent years extensive genotypic studies have been performed on 250 *Bradyrhizobium* strains from *Aeschynomene*, *Faidherbia albida* and diverse small legumes in Senegal, using several techniques i.e. 16S rDNA RFLP and sequence analyses, 16S-23S rDNA intergenic gene spacer RFLP and sequence analyses, AFLP, DNA:DNA hybridisations [14, 15, 64, 66, 67]. Several genospecies were evidenced, at least 8 of which were distinct from the three recognised species [67].

4. CONCLUSION

From one genus and 6 species in 1982, taxonomic studies of rhizobia have led to a total of 6 genera and 28 recognised species. This is in constant progress due to progressive study of uninvestigated legumes and areas in the world. Progress in taxonomy is also due to an increasing number of available effective techniques in bacterial characterisation, more and more accessible to an increasing number of laboratories. Only 23% of the total number of legume species (that is between 16.500 and 19.500) have been characterised for their microsymbionts so far. The majority (88%) of these investigated legumes were found to be nodulated. Specifically, tropical rhizobia are still poorly documented compared to what can be expected from the recent data, showing great diversity in China, Brazil, Senegal, Sudan, Morocco. Recently, Sy et al. [53] reported that some rhizobia isolated from *Crotalaria* spp. in Senegal were phylogenetically separated from all recognised rhizobia. They form a new species in the *Methylobacterium* genus, for which the name *M. nodulans* has been proposed. This predicts that several other new groups of legume symbiotic bacteria (genera, species) may emerge in the future.

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