TAXONOMY AND PHYLOGENY OF DIAZOTROPHS

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1. Introduction

The biodiversity of nitrogen-fixing organisms is huge. Taxonomic and phylogenetic research is needed to structure this diversity, to facilitate communication among scientists, and to increase our understanding of the evolution and biology of diazotrophs. Molecular tools for taxonomic and biodiversity studies of diazotrophic rhizobia, frankiae, cyanobacteria and bacilli are presented in sections 2 to 5. Sections 6 to 9 focus on problems with genus and species assignment.

2. Molecular tools for *Rhizobium* taxonomy and biodiversity studies.

The current taxonomy and phylogeny of rhizobia is based on the study of bacteria isolated from nodules of few legume species compared to the large diversity of legumes (estimated at up to 18.000 species). Nevertheless, the current taxonomy of the legume symbionts reveals a large biodiversity at the genus, species and intraspecies levels. Single legume species are often nodulated by different bacterial species or genera and most rhizobia can nodulate several legume species and genera. Recent reviews on phylogeny, taxonomy and genetic diversity of rhizobia have been published (Young, Haukka, 1996; Martinez-Romero, Caballero-Mellado, 1996). With the development of molecular methods, a battery of molecular tools is currently available to analyze the variability of the bacterial genome and to assess genetic relationships among bacteria.

Sequence comparisons of full length 16S rRNA genes is now the standard method for assessing phylogenetic relationships among bacteria. Applicability of the method was considerably improved with the development of PCR technology and a large database of sequences is now available. Recent subdivisions of the genus *Rhizobium* into two new genera, *Sinorhizobium* (de Lajudie et al., 1994) and *Mesorhizobium* (Jarvis et al.,

C. Elmerich et al. (eds.), Biological Nitrogen Fixation for the 21st Century, 559-570.
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unpublished) was guided by sequence analysis of 16S rRNA genes. However, the method lacks of sensitivity to discriminate between phylogenetically closely related species. The analysis of the larger 23S subunit of rRNA may improve the resolution power at the species level (Ludwig et al., 1995) and some partial rhizobial sequences have been recently determined (Selenska-Pobell, Evguenieva-Hackenberg, 1995). A strategy of sequencing only a 300 bp variable region of 16S rRNA genes (Young et al., 1991; Oyaizu et al., 1992; Haukka et al., 1996) has been used, but the resulting phylogenies are not congruent with those from the complete sequences for certain rhizobial species (Nour et al., 1995; Young, Haukka, 1996). Another strategy, based on restriction fragment length polymorphism (RFLP) analysis of the full-length PCR-amplified 16S rDNA, has been used (Laguerre et al., 1994). The topology of the phylogenetic trees obtained from mapped restriction sites and from aligned sequences were well correlated, showing that the method is powerful for a rapid estimate of phylogenetic relationships (Laguerre et al., unpublished).

DNA relatedness is the standard arbiter for drawing species boundaries. However, the species boundaries are not always clearly delineated. DNA similarity values should not be lower than 70% within a single species and low similarity values with all previously described species should support the creation of a new species. The problems with this guideline are that intermediate values (50 to 65%) occur as in the *Mesorhizobium* genus (see, for example, Sullivan et al., 1996) and that values may vary with the technique used. Also, the method is laborious and the number of recognized species of rhizobia is increasing rapidly. Therefore, the method is often applied to representative strains within a group of rhizobia delineated by more rapid methods and could be limited to comparisons of groups with very similar 16S rDNA sequences.

At the intraspecies level, diverse methods based on DNA or protein analysis by gel electrophoresis have been used for typing and clustering rhizobial isolates. There are methods which provide fingerprints of the overall genome and methods which detect variability in specific gene regions. The latter provide information on the evolution and the genetic structure of natural populations, including genetic recombination and horizontal gene transfer. The first-category methods include analysis of whole-cell protein patterns and of RFLP of the whole-genome, and more recent techniques of PCR DNA fingerprinting based on simultaneous amplification of multiple DNA fragments using random primers (RAPD) or repetitive (rep) sequences as primers. These methods are universal for all bacteria and highly discriminating at the intraspecies level. However, they have the disadvantage of generating complex patterns which have to be standardized to obtain reproducible fingerprints. The second-category methods include multilocus enzyme electrophoresis (MLEE). RFLPs using DNA probes, and sequencing or RFLP analysis of PCR-amplified DNA regions. The level of resolution depends on the number of molecular markers used and on the genetic region analyzed. Genetic relationships can be estimated by numerical analysis when enough markers or restriction enzymes are used. MLEE is an universal method to assess genetic diversity of natural populations, but it reveals mainly the variability of the chromosome. Various DNA probes and oligonucleotide PCR primers homologous to either chromosomal or plasmid DNA regions, including symbiotic genes, have been used to type rhizobia. The specificity depends on the target DNA region and the hybridization conditions. Probes universal for all the bacteria or the nitrogen-fixers or the rhizobia can be prepared from conserved DNA regions and 16S rDNA or from nitrogenase structural genes or from common *nod* genes, respectively. Different PCR-based methods, which include PCR DNA fingerprinting, PCR-RFLP of 16S-23S rDNA spacer regions, *nif* and *nod* gene regions have been recently compared for their respective levels of resolution (Laguerre et al., 1996). With the development of DNA probes for colony hybridization,

diversity of rhizobial populations isolated from soil samples has been recently reported (Bromfield et al., 1995; Louvrier et al., 1996). Chromatographic profiling of Nod factors was recently proposed by Lortet et al. (1996) as an easy and powerful tool for classification of rhizobia on the basis of their symbiotic properties.

3. Phylogeny and population biology of *Frankia* spp.

The taxonomy of *Frankia* has been debated for decades. Host plant infectivity and symbiotic morphology have been two phenotypes studied but they have been misleading. DNA/DNA homology has finally shown that there were several genomic species in the group of strains infective on *Alnus* (3 species) and in the group of strains infective on Elaeagnaceae (7 species) but only one in the group of strains infective on *Casuarina* (Fernandez et al., 1989).

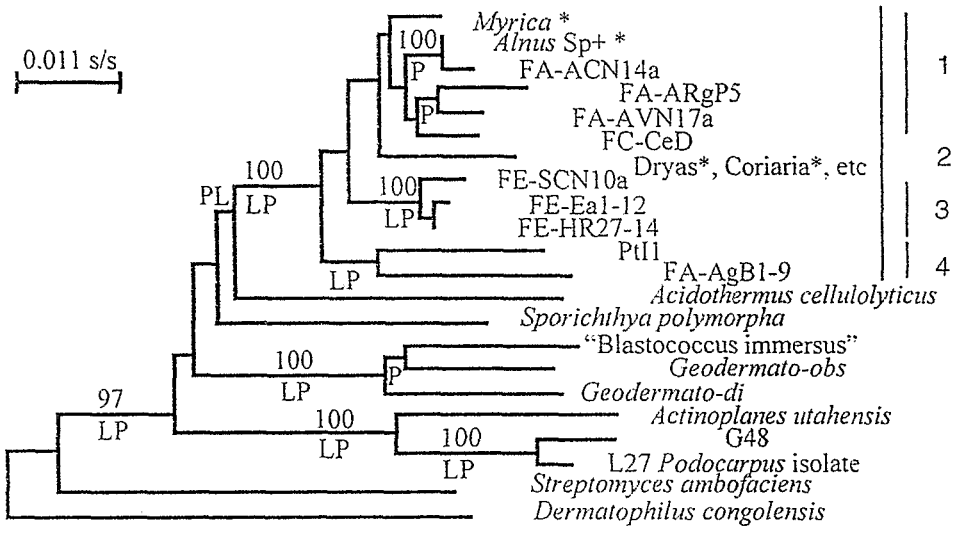

Figure 1. Phylogenetic tree of *Frankia* and phyletic neighbors according to Normand et al. (1996). The four clusters are indicated on the right, the numbers besides nodes indicate the % of bootstrap replicates, the « P » and « L » indicate the groupings detected by Parsimony analysis and Maximum Likelihood, respectively.

To study the relation of these genomic species, the 16S rDNA sequence was determined for several *Frankia* strains, either isolated or not, and for other actinomycetes with a similar morphology belonging to genera *Geodermatophilus*, *Blastococcus* and *Dermatophilus*. The different *Frankia* strains were grouped into 4 clusters: 1, strains infective on *Alnus* (Betulaceae), *Casuarina*, *Allocasuarina* (Casuarinaceae) and *Myrica* (Myricaceae); 2, unisolated strains present in nodules of *Dryas* (Rosaceae), *Coriaria* (Coriariaceae) and *Datisca* (Datiscaceae); 3, strains infective on Elaeagnaceae and *Gymnostoma* (Casuarinaceae); and 4, a group of strains isolated from diverse host plants but uninfective or infective (but *nif*$^-$) on *Alnus* (Figure 1). Contrary to what was expected, the closest *Frankia* neighbors were not *Geodermatophilus* and *Blastococcus* but the bacterium, *Acidothermus cellulolyticus* (Normand et al., 1996). These sequences were used for a specific primer for

the *Alnus* rhizosphere to study the *Frankia* populations by a PCR/cloning/RFLP approach, however, the primer was not discriminating enough (Normand, Chapelon, 1997).

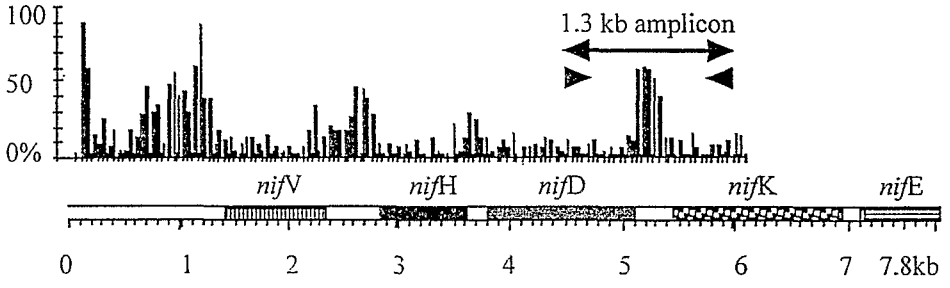

Figure 2. Histogram of differences in the *nifVHDK* genes between *Frankia* strains ArI3 and FaC1. Arrows indicate position of the primers used in the nitrogenase structural subunits genes framing the 1.3 kb amplicon restricted to directly analyze nodule strains.

Because of the presence in the rhizosphere of non-symbiotic close phyletic neighbors, the *nifDK* intergenic spacer has been targeted (Figure 2) and used with *Gymnostoma* spp. (Casuarinaceae) nodules. The bacteria present were close to Elaeagnaceae-infective *Frankia* but not to other Casuarinaceae-infective strains (Navarro et al., 1997). This result supports the idea that coevolution has not occurred in *Gymnostoma*-infective strains contrary to what is found in *Casuarina* and *Allocasuarina*-infective strains (Rouvier et al., 1996) where a strict pattern of correspondence was found between *Frankia* strains (IGS-type) and the 5 species of *Casuarina* and *Allocasuarina* strains.

4. Diversity studies of cyanobactria using STRR and LTRR sequences for PCR.

Cyanobacteria (blue-green algae) are unique among prokaryotic organisms in possessing oxygenic photosynthesis and nitrogen fixation within the same organism. Thus, cyanobacteria are the most successful and wide-spread group among the prokaryotes and they are found in diverse terrestrial and aquatic environments where they form natural symbioses with Bryophyta (mosses, liverworts and hornworts), gymnosperms of the family Cycadaceae, an aquatic fern within Pteridophyta (*Azolla*) and an angiosperm, *Gunnera*, as well as diverse lichenized fungi. With few exceptions, the symbiotic cyanobacteria belong to the filamentous genus, *Nostoc* (Rippka et al., 1976). Reconstitution experiments with the *Anthoceros-Nostoc* and *Gunnera-Nostoc* symbioses have shown that some *Nostoc* can form a symbiosis with both hosts (Bonnett, Silvester, 1981; Enderlin, Meeks, 1983; Johansson, Bergman, 1994). These results raise interesting questions concerning the specificity and diversity among symbiotic *Nostoc*, both within and among different host plants. Using protein profiles and RFLP techniques on a few *Nostoc* isolates from *Gunnera* and cycads (Lindblad et al., 1989; Zimmerman, Bergman, 1990; Zimmerman, Rosen, 1992), diversity has been shown to exist among the symbiotic isolates from different plant species.

The use of rep-PCR (de Bruijn, 1992) and DNA from 14 axenic cyanobacterial cultures (symbiotic and free-living) has produced both ERIC and REP-PCR fingerprints, indicating

that ERIC and REP primers may be used for fingerprinting axenic cyanobacterial cultures. Recently, the presence of repetitive sequences, which are specific for cyanobacteria, the so-called short tandemly repeated repetitive (STRR) and long tandemly repeated repetitive (LTRR) sequences (Mazel et al., 1990; Jackman, Mulligan, 1995; Masepohl et al., 1996) have been identified. SLRR were identified in a number of heterocystous cyanobacterial genera and species (Mazel et al., 1990). In *Calothrix* PCC 7601, the copy number was estimated to about 100 per genome. LTRR sequences, 37 bp long, were found in both heterocystous and non-heterocystous cyanobacteria and, in *Anabaena* PCC 7120, was found in 10 regions of the genome (Masepohl et al., 1996). Thus, primers, corresponding to the STRR and LTRR sequences and used in PCR reactions, were shown to be selective for cyanobacteria and to generate a specific fingerprint for individual isolates, using either purified DNA and intact cyanobacterial cells as templates. Thus, it is a very efficient method of identifying cyanobacteria in complex microbial populations.

In 35 cyanobacterial isolates for analysis, 23 were *Nostoc* collected from different species of the angiosperm *Gunnera*. The results of both LTRR-PCR and STRR-PCR revealed both a high genetic diversity among the isolates and a distinct clustering of 15 isolates into four groups. Thus, the individual isolates in each group must be considered as identical or closely related. An axenic isolate, *Nostoc* PCC 9229, showed the same fingerprint pattern as four non-axenic isolates indicating the specificity of the PCR method for fingerprinting cyanobacteria from non-axenic cultures. Moreover, isolates from different *Gunnera* species revealed the same fingerprint pattern, which substantiates earlier observations that one *Nostoc* strain can form a symbiosis with different *Gunnera* species.

5. Firmicutes: N_2 fixation occurs in a monophyletic subset of *Paenibacillus* species.

Several species of the aerobic endospore-forming Firmicutes, including *Bacillus polymyxa*, have been recently reclassified into the new genus, *Paenibacillus* (Ash et al., 1993). According to *rrs* (RNA 16S gene) sequence data, the three major N_2-fixing species formed a monophyletic cluster in *Paenibacillus* and were renamed *P. azotofixans*, *P. polymyxa* and *P. macerans*. A fourth species, including some N_2-fixing strains of *B. circulans*, which were described as phenotypically heterogeneous, remained in genus *Bacillus* on the basis of *rrs* sequence analysis of its type strain (ATCC 4513^T). Phenotypic identification of strains PMD 230 and CF43 as *Paenibacillus polymyxa* was confirmed with *rrs* sequencing. However, phenotypic identification (API 50CH; Berge et al., 1991) of strains RS19 and TOD45 as *Bacillus circulans* (group 2), which were isolated from the rhizosphere of wheat and maize, was not confirmed by *rrs* sequence data. These two sequences were found to form a monophyletic cluster with *Paenibacillus azotofixans*, *P. polymyxa* and *P. macerans*. They constitute a new species, which will probably include part of the formerly named *B. circulans* 2. Corrections within *B. circulans* started recently with the description of *P. illinoisensis* (Shida et al., 1996) for which N_2 fixation has to be assessed. Based on *rrs* sequence data, *B. longisporus* has to be transferred into *Paenibacillus* genus together with taxonomically related *P. amylolyticus* and *P. pabuli*.

The N_2-fixation capacity of *P. azotofixans*, *P. polymyxa*, *P. macerans* and strains TOD45 and RS19 was confirmed by *nifH* sequencing and ARA measurements. The phylogenetic tree of *nifH* sequences (Figure 3) was compared to the *rrs* sequence tree. All *Paenibacillus nifH* sequences formed a coherent cluster, away from the closely related anaerobic *Clostridium* and the Gram-positive high G+C content *Frankia*. *nifH* was detected in neither *B. circulans* type strain (ATCC 4513^T) nor *B. subtilis*, *B. cereus*, *B. brevis*, *B. alcalophilus* and *B. simplex* type strains. This result suggests that, among aerobic endospore-forming Firmicutes, N_2 fixation is restricted to genus *Paenibacillus*.

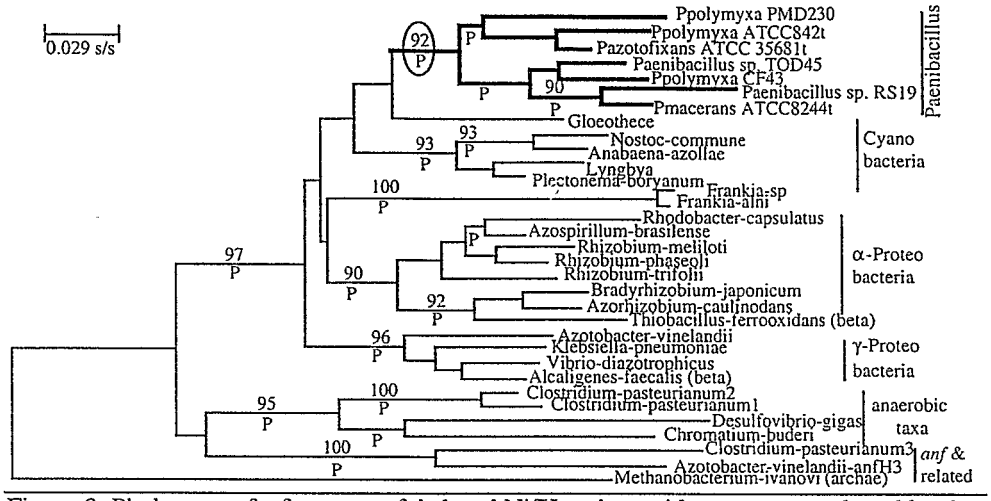

Figure 3. Phylogeny of a fragment of deduced NifH amino-acid sequences analyzed by the NJ method (Saitou, Nei, 1989). The % of bootstrap replicates (Felsenstein, 1985) that support each group are indicated above the nodes and those supported by parsimony (Kluge, Farris, 1969) are indicated by « P » below it.

6. Origins of *Mesorhizobium* gen. nov.

Lotus pedunculatus (Big trefoil) and *L. corniculatus* (Birdsfoot trefoil) have been promoted as legume species for low fertility pastures where they appear more persistent and higher yielding in dry matter than white clover cultivars (Seaney, Henderson, 1970). Both fast- and slow-growing *Lotus* rhizobia were recognized and fast-growing *Lotus* rhizobia were described as a new species (Jarvis et al., 1982). At this time, *R. loti* was well established as a member both of an extensive cross-inoculation group on *Lotus, Anthyllis* and *Astragalus* spp. and of a DNA homology group, which includes strains from a variety of species. The following developments support the decision to re-classify *R. loti.*

Several new species related to *R. loti.* have been recently introduced, including *R. huakuii* (Chen et al., 1991) from *Astragalus sinicus*; *R. ciceri* (Nour et al., 1994), *R. mediterraneum* (Nour et al., 1995) from *Cicer arietinum*, chickpeas, and *R. tianshanense* (Chen et al., 1995). The position of the latter is somewhat controversial from genomic DNA relatedness studies. Recently, Entao Wang re-examined the relationship between *R. tianshanense* and the type strains of related species using ^{32}P-labeled probe DNA from strains A-1BS on Southern blots from other strains. DNA relatedness values between *R. tianshanense* and *R. huakuii, R. loti* and *R. mediterraneum* were 18, 13, 4, and 43%, respectively. These values suggest that *R. tianshanense* is a separate and probably related species. Further, the relationship between *R. loti* and other *Rhizobium* and *Sinorhizobium* species was clarified by rDNA:DNA hybridization studies (Jarvis et al., 1986) and it was concluded that *R. loti* was more distant from *R. leguminosarum* than *Agrobacterium* and *Sinorhizobium* sp. Subsequent SS rRNA gene sequencing studies (Willems, Collins, 1993) have confirmed that *R. loti* is remote from other *Rhizobium* species and contributes to the current polyphyletic character of the genus. Whole-cell fatty

acid analysis (Jarvis et al., 1986) indicates that the "*R. loti* group" possesses unusual fatty acids making them phenotypically distinguishable from other rhizobia.

Thus, the "*R. loti* group" is phenotypically and phylogenetically distinguishable from other rhizobia and has diverged sufficiently from other rhizobia to constitute a separate genus. The genus name, *Mesorhizobium* , was initially proposed by Chen et al. (1995) to indicate a growth rate intermediate between that of *Bradyrhizobium* strains and typical fast-growing *Rhizobium* strains. Subsequently, it was used to denote a phylogenetic position (Young, 1996) for rhizobia intermediate between these two genera.

7. Polyphasic taxonomy and species delineation in *Mesorhizobium*.

Until 1991, *R. loti* was the only member of the separate *R. loti* rRNA branch. Since then, several new species and groups have been described on this rRNA branch (see above), including cluster U (de Lajudie et al., 1994) and *Rhizobium* sp. (*Cicer*) genomic group 3 and 4 (Nour *et al.*, 1995). At the 10th International Congress on Nitrogen Fixation, a new genus, *Mesorhizobium,* was proposed to group all the species of the *R. loti* rRNA branch (Lindström et al., 1995; Jarvis et al., unpublished). Several other emerging rhizobium groups also belong to *Mesorhizobium* (Nick et al., 1995; Novikova et al., 1995; Limeschenko et al., 1995; Wang, Martinez, personal communication). However, specific taxonomic problems inside *Mesorhizobium* have arisen, for example, in the complex group U (de Lajudie et al., 1994).

Cluster U was first identified as a separate cluster both by protein gel electrophoresis and auxanography. Since then, more isolates and additional reference strains of the *Mesorhizobium* rRNA branch have been included in the SDS-PAGE analysis. Cluster U, now comprising 35 strains from different leguminous species in Africa and Brazil, is clearly distinct from other species of related genera, such as *Rhizobium, Azorhizobium, Sinorhizobium,* and *Bradyrhizobium* and also from the different *Mesorhizobium* species included in the study. Four subclusters (Ua, Ub, Uc, Ud) can be discerned. *M. loti* strains were found in different places in the dendrogram. Two *M. loti* strains, LMG 6123 and LMG 6124, formerly considered to belong in cluster U, now group outside it. Despite the internal heterogeneity of the SDS-PAGE cluster and previous evidence of different genotypic subgroups (de Lajudie et al., 1994), 16S rRNA gene sequences of representative members of the different subclusters of cluster U were found to be identical. Their common sequence showed 98% sequence similarity with *M. loti* and *M. ciceri* (25 differences in 1,436 bases), 99.6% (6 differences in 1,430 bases) with *M. huakuii* , and 98.7% (17 base differences) with the recently proposed species *M mediterraneum* (Nour et al., 1995). These results clearly indicate that cluster U strains form a separate lineage on the *Mesorhizobium* rRNA branch. The sequences of the two strains, LMG 6123 and LMG 6124, were different from each other (99.7% sequence similarity), from the type strain of *R. loti*. (98.0-98.3% sequence similarity), from cluster U (99.4% sequence similarity), and from *M. huakuii* (99.8% sequence similarity). The high phylogenetic homogeneity inside cluster U was further confirmed by 16S rDNA gene PCR-RFLP on a selection of 16 strains exhibiting identical profiles with 6 different enzymes (Nick et al., personal communication). Extensive DNA:DNA hybridization experiments revealed a certain degree of internal genetic heterogeneity inside cluster U. Two main genotypic groups (groups I and II) with high internal DNA hybridization values (average 83% inside each and 39% between the groups) were found plus several other intermediate strains with a mean DNA homology value of 38 % with other cluster U strains. Group I consists of senegalese and sudanese strains and corresponds mainly to clusters Ua and Ub, whereas

Group II consists of senegalese and brazilian strains from cluster Uc. DNA-binding was not significant between strains LMG 6123 and LMG 6124 (38%) and between either of these strains and any other strain tested on the *Mesorhizobium* rRNA branch. Rep-PCR experiments, using BOXA1R, GTG5, REP or ERIC primers, on representatives strains matched the results of DNA:DNA hybridization.

In accordance with the guidelines for the definition of species (Wayne et al., 1987), it could be argued that a new *Mesorhizobium* species should be created for each of these genospecies because they can be distinguished phenotypically. However, this is not advisable but we do propose to create a single new species, *M. plurifarium* , for these strains as occurred for *R. tropici* where two genospecies (type A and type B) could be distinguished. This decision was later validated when *R. tropici* strains intermediate between type A and type B were described exhibiting characteristics of both types (Martinez-Romero, 1996). Our results bring additional evidence that *M. loti* is definitely heterogeneous and needs revision according to modern taxonomic criteria. It should be pointed out that a polyphasic approach is even more important when dealing with *Mesorhizobium* strains than in other groups.

8. New rhizobial groups and remarks on type strains.

In spite of the large number of legume species, relatively few rhizobial species have so far been described (Martinez-Romero, Caballero-Mellado, 1996). It is quite likely that the isolation of bacteria from nodules of non-explored legume species will provide new (*Sino*) (*Meso*) (*Brady*) (*Azo*) *Rhizobium* species, but these isolates do not always represent a sequence cluster. Various possibilities may explain this (Martinez-Romero, Caballero-Mellado, 1996). SSU rRNA genes, which constitute the normal basis for the definition of a sequence cluster, change too slowly to account for the genetic divergence that has occurred recently and that may be driven in part by acquisition or loss of genetic modules (plasmids, or even parts of chromosomes) (Sullivan et al., 1995). Comparison of other gene sequences as well as of chromosomal genetic maps may provide higher degree of discrimination in the analysis of the diverging branches in rhizobial phylogenies. With this in mind, we propose the sequences of glutamine synthetase genes (GSII) and citrate synthase genes from different *Rhizobium* species for future phylogenic analysis.

Relaxed specificity, normally encountered in many tropical legumes, allows more than one bacterial species can often be isolated from nodules of a single legume. A wide range of genetically distinct bacteria have been isolated from bean nodules (Eardly et al., 1995; Hernandez-Lucas et al., 1995; Martinez-Romero et al., 1991; Segovia et al., 1993; Martinez-Romero, 1996). This also occurs with *Leucaena leucocephala*, *Amorpha fructicosa* , *Acacia* (de Lajudie et al., 1994) and *Sesbania* (Boivin et al. 1997). Five ribotypes were identified by PCR-RFLPs among 55 *Amorpha Rhizobium* strains. We propose *Mesorhizobium amorphi* as a new species for ribotype 1 isolates, which includes the majority of *Amorpha* symbionts. These species harbor a 900 Kb megaplasmid carrying nodulation and nitrogen-fixing genes. *Mesorhizobium* species (Jarvis et al., unpublished) seem to show a high degree of similarity in their SSU rRNA genes, and this may indicate that they have all radiated recently from a common ancestor. In contrast, the isolation of symbionts from *Sesbania herbacea* in Mexico provided us with a genetically narrow group, which was related to *Rhizobium galegae* but distinguishable from it by total DNA-DNA homology, its plasmid profiles, its host range and other phenotypic traits (Table 1). If this group represents a new species, it should constitute a new genus together with *R. galegae* (Lindstrom, 1989).

Table 1. Differences between *R. galegae* and *Rhizobium* sp. (*Sesbania herbacea*).

	R. galegae	*Rhizobium* sp.(*Sesbania herbacea*)
Phenotypic traits		
Growth in YAM at 37 to 40C	-	+
at pH9.0	-	+
Resistant to ampicillin (5 $g.ml^{-1}$)	-	+
Sole nitrogen source		
adenine	-	+
methionine	-	+
Sole carbon source		
dulcitol	-	+
lactose	-	+
DNA homology (%)		
to HAMBI503 (*R. galegae*)	35-100	12-18
to S02 (*Sesbania herbacea*)	14-22	78-100
DNA G+C mol %		57-58
Plasmids (Kb)*	1100,300,170	900,400,200
nif-nod plasmid	1100	400
Sesbania nodulation	-	+
Galega nodulation	+	-
MLEE groups	I,II	III

*Different strains harbor different plasmids. Some *R. galegae* strains harbor only the 1100 Kb plasmid, some harbor 1100 and 300 Kb plasmids, others harbor 1100 and 170 Kb plasmids and few strains have no plasmids or harbor two very small (<50 Kb) ones.

Essential in the systematics and taxonomy of any bacterium is the availability of type reference strains, which should be good representatives of a species, however, problems exist with several rhizobial reference strains. For example, there are subcultures of the *B. japonicum* type reference strain in many collections all over the world but it has at least ten synonyms! It was originally introduced into the USDA *Rhizobium* collection in 1929 as USDA 6 and it was provided by USDA to ATCC in 1948 (Barrera et al., unpublished). The SSU sequences of two type strains, IAM 12608 and LMG 6138, were previously reported (accession numbers D11345 and X66024). They differed by 11 nucleotides. The sequence of IAM 12608 is similar to that of the *B. japonicum* group 1a strain, USDA 110, so it is possible that the sequence of IAM 12608 was that of a contaminating *B. japonicum* strain rather than the type strain. Recent re-sequencing of USDA 6 found that it is very similar to LMG 6138, which, according to SSU rRNA gene, seems to be a very good representative of the *B. japonicum* species because its SSU rRNA gene sequence agrees closely with the *B. japonicum* consensus sequence. In addition, there are two versions of *R. tropici* CIAT 899 distributed in different labs. One is more competitive for nodule formation and the two also differ in the size of the nodulation plasmid (Romero, personal communication). The two reported partial sequences (300 bp) of *Azorhizobium* 16S rRNA genes (accession numbers X67221 and M55491), which are allegedly both from the type strain, are only 84% homologous; plus different *M. loti* type strains have shown up. If original names for the type strains had been retained, then perhaps less confusion would exist in regard to type strains. Further comparison of type reference strains are required (for example, in the case of *A. caulinodans*) to ascertain the reasons for the discrepancies.

9. Polyphasic taxonomy of a new rhizobial group from the Loess Plateau of China.

The Loess Plateau, well known for its deep deposit and wide distribution of loess is located in the northwest of China. Severe erosion has transformed it into a hilly-gully area of silty loam, both fragile and porous, with a pH of 8.4 plus 18% $CaCO_3$ and 0.45% organic matter. Thus, the floral rate is very low, but some stress-tolerant legumes and other plants can grow there. Previously (Tan et al., 1997), three DNA homology groups of rhizobia were isolated from the various wild legumes on the Loess Plateau and found to be different from other species of root-nodule bacteria. Recently, strains have again been isolated from the same host plants and one group (cluster 9) has been studied in detail. 34 bacterial strains were used, among which 23 were isolated from *Coronilla varia*, nine from *Amphicarpaea trisperma* and two from *Gueldenstaedtia multiflora*..

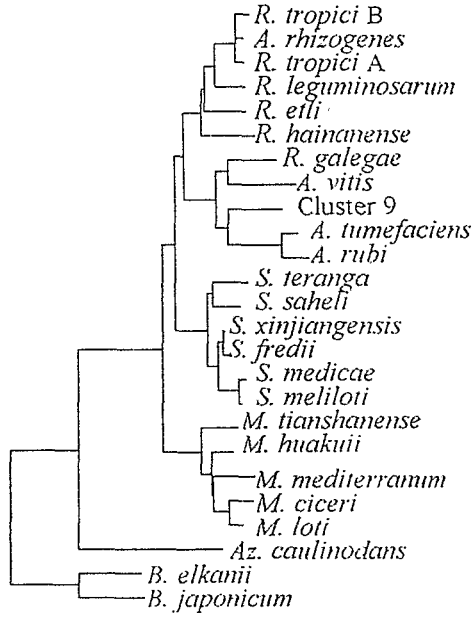

Figure 4. Dendrogram showing the relationships of rhizobia and related bacteria.

All 34 rhizobial strains formed a single cluster (cluster 9, by UPGMA clustering, above the similarity level of 82%) on the basis of SDS-PAGE whole-cell protein patterns. The G+C content within cluster 9 was 59.7-63.1 mol%, which places the cluster within the rhizobia (Graham et al., 1991). The level of DNA relatedness between the representative strain SH22623 of cluster 9 and the other members was 71.3-93.5 %. The DNA homologies between strain SH22623 and type strains of known rhizobial species was 14.5-59.0 %. These results showed that cluster 9 forms a DNA homology group with higher than 70 % homology, which is distinct from the recognized rhizobial species. The 16S rDNA sequence of strain SH22623 has been deposited in the EMBL and BanKIT sequence databases under accession number AFOO3375. Comparing known rhizobial and agrobacterial sequences gave similarities between cluster 9 and *Agrobacterium tumefaciens*.

A. rubi, A. vitis and *Rhizobium galegae* of 96.0%, 95.4%, 93.4% and 93.8%, respectively. The dendrogram (Figure 4) shows that cluster 9 belongs to the *Agrobacterium-R. galegae* phylogenetic branch and so cluster 9 forms a genotypically and phenotypically distinct group of root-nodule bacteria.

10. Acknowledgments.

P. Normand acknowledges the coauthorship of S. Orso, B. Cournoyer, C. Rouvier, P. Simonet and M. Fernandez, Lyon, France, of E. Navarro and Y. Prin, LSTM, Montpellier, France, and of P. Reddell, CSIRO, Townsville, Australia; U. Rasmussen that of Mette M. Svenning, University of Tromsø, Norway; T. Heulin that of W. Achouak, Cadarache, and P. Normand, Lyon, France; E. Martínez-Romero that of E.-T. Wang, I. Hernandez-Lucas and D. Romero, and gratitude to Marco A. Rogel for technical support and for DGAPA grant from UNAM; P. de Lajudie acknowledges the coauthorship of A. Willems, B. Hoste, U. Torck, K. Kersters, and M. Gillis, Gent, Belgium, of Molouba, M. Neyra and B. Dreyfus, Dakar, Senegal, and of G. Nick and K. Lindström, Helsinki, Finland; W.-X. Chen acknowledges the coauthorship of Z. Tan and financial support from National Natural Science Foundation of China.

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P. de LAJUDIE

Biological Nitrogen Fixation for the 21st Century

Proceedings of the 11th International Congress on Nitrogen Fixation,
Institut Pasteur, Paris, France, July 20–25, 1997

edited by

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