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Recent developments in *Rhizobium* taxonomy

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Abstract

Recent developments in *Rhizobium* taxonomy are presented from a molecular and evolutionary point of view. Analyses of ribosomal RNA gene sequences provide a solid basis to infer phylogenies in the Rhizobiaceae family. These studies confirmed that *Rhizobium* and *Bradyrhizobium* are only distantly related and showed that *Rhizobium* and *Bradyrhizobium* are related to other groups of bacteria that are not plant symbionts. *Rhizobium* and *Agrobacterium* species are intermixed. Differences in plasmid content may explain to a good extent the different behavior of *Rhizobium* and *Agrobacterium* as symbionts or pathogens. Other approaches to identify and classify bacteria such as DNA-DNA hybridization, fatty acid analysis, RFLP and RPD-PCR techniques and phylogenies derived from other genes are in general agreement to the groupings derived by ribosomal sequences. Only a small proportion of nodulated legumes have been sampled for their symbionts and more knowledge is required on the systematics and taxonomy of *Rhizobium* and *Bradyrhizobium* species.

Introduction

New molecular approaches that analyze the bacterial genome are renewing our interest in bacterial systematics and taxonomy, and broadening the perception that man has of microbes. These approaches have not only revealed unsuspected relationships among apparently unrelated bacteria, but also demonstrated the existence of marked genetic diversity within groups of microorganisms. These analyses could help to understand mechanisms that operate in bacterial evolution, they provide tools to confidently identify bacteria, and also provide a solid reference framework for other type of studies.

DNA-DNA or DNA-RNA hybridization, restriction fragment length polymorphism analysis of DNA (RFLP), RPD's (de Bruijn, 1992), DNA sequencing as well as other approaches such as the electrophoretic analysis of metabolic enzymes and numerical taxonomy have proven valuable in bacteria taxonomy and systematics (Selander et al., 1986; Schleifer and Stackebrandt, 1983). Both the conservation of ribosomal RNA due to its structural constraints in ribosomes, and the existence of variability in some domains, render ribosomal RNA genes sequences (5S, 16S, and 23S) as very good choices to compare organisms and to infer phylogenies (Woese, 1987).

Phylogenies of the Rhizobiaceae based on 16S rRNA sequences

Bradyrhizobium, *Azorhizobium* and *Rhizobium* species form nodules in the roots or stems of

legumes where they fix atmospheric nitrogen. These species and their host plants are listed in Table 1. Analysis of 16S ribosomal sequences revealed that *Rhizobium* and *Bradyrhizobium* are only distantly related, but that each has close relationships to other groups of bacteria that are not plant-symbionts (Sawada et al., 1993; Willems and Collins, 1993; Yanagi and Yamasato, 1993; Young et al., 1991). *Bradyrhizobium* spp., including the phototrophic strain BTail are more related to *Rhodopseudomonas*, to *Afipia* and to *Blastobacter denitrificans*. (Willems and Collins, 1992; Young et al., 1991). *Rhizobium* is related to *Agrobacterium*, to *Brucella*, to *Rochalimea* and to *Bartonella*. *Phyllobacterium*, one of the other genera of the Rhizobiaceae that forms hypertrophies on leaves, also appears related to *Rhizobium huakuii* and to *R. loti* (Yanagi and Yamasato, 1993).

A summary phylogenetic tree is shown in Fig. 1 which gathers data from published (Sawada et al., 1993; Willems and Collins, 1992; Yanagi and Yamasato, 1993; Young et al., 1991) and unpublished (Hernández-Lucas et al.) genetic distances derived from partial sequences and from full sequences of 16S rRNA genes.

Some differences in phylogenetic trees may be obtained depending on the theoretical analysis performed. Contrast, for example, the Fitch-derived tree and the parsimony analysis- tree derived by Willems and Collins (1993). Some branches of phylogenetic trees can be predicted with high probabilities. For others, alternate node positions having equal probabilities may be possible, making their positions uncertain.

Agrobacterium and *Rhizobium* species are consistently intermingled (Sawada et al., 1993; Willems and Collins, 1993, Yanagi and Yamasato, 1993). *R. galegae* is a branch among other agrobacteria lineages, while the degree of relationship between *R. tropici*, *Agrobacterium rhizogenes* and *Agrobacterium* spp. is remarkable. *R. tropici* is native to South America but has also been encountered in nodules of *P. vulgaris* from acid soils in Kenya (Giller, unpublished). It is a broad host rhizobia that nodulates *P. vulgaris* bean, *Leucaena* spp. and some other legumes (Martínez et al., 1993); *R. tropici* is able to grow in acid pH, and is tolerant to aluminum (Graham et al., 1982), and of high temperatures. *R. tropici* has been subdivided in two types based on phenotypic differences and differences in ribosomal RNA genes (Martínez et al., 1991). *Agrobacterium* sp. K-Ag-3 was isolated from a tumor from a Kiwi plant in Hiroshima, Japan and Ch-Ag-4 was isolated from cherry in Okayama, Japan (Sawada and Ieki, 1992) and according to these authors they represent two subtypes of unclassified agrobacteria. By the analysis of complete ribosomal 16S RNA genes, K-Ag-3 is indistinguishable from *R. tropici* type B and very close to Ch-Ag-4 and to *R. tropici* type A. Interestingly, the similarities in ribosomal sequences between *Rhizobium* and *Agrobacterium* are in agreement with similarities in colony morphology and growth in different media. *R. tropici* strains do not form tumors in sunflower (Martínez, unpublished). As acid resistance is determined chromosomally in *R. tropici*, (P. Graham, personal communication), we tested Ch-Ag-4 and K-Ag-4 for growth in acid medium. These agrobacteria were able to form colonies in MM in pH 4.5. Differences in plasmid content may explain to a good extent the different behavior of *Rhizobium* and *Agrobacterium* as symbionts or pathogens. Thus, it is important to distinguish between the evolutionary histories of plasmids and the evolutionary histories of chromosomes in phylogenetic studies of bacteria.

Phylogenies of plasmids and evidence of their transfer

In *Rhizobium*, most symbiotic information lies on extrachromosomal elements termed symbiotic (sym) plasmids (Martínez et al., 1990), but other plasmids have also been shown to be involved in the symbiotic process (Brom et al., 1992; Hynes and McGregor, 1990; Martínez and Rosenblueth, 1990). In *Agrobacterium*, plasmids are responsible for tumorigenesis. Non-symbiotic rhizobia (Segovia et al., 1991) and non-pathogenic agrobacteria (Kersters and de Ley, 1984) have been described. Non-symbiotic rhi-

Table 1. Species of root and stem-nodulating bacteria and their hosts

Rhizobium species:	Host legumes:
Rhizobium meliloti	*Medicago, Melilotus, Trigonella*
Rhizobium fredii, R. xinjiangensis	*Glycine max* and *G. soja* and other legumes
Rhizobium leguminosarum	
bv. viciae	*Pisum, Vicia*
bv. *trifolii*	*Trifolium*
bv. *phaseoli*	*Phaseolus*
Rhizobium tropici	*Phaseolus vulgaris, Leucaena* spp.
Rhizobium etli	*Phaseolus vulgaris*
Rhizobium galegae	*Galega officinalis, G. orientalis*
Rhizobium loti	*Lotus* spp.
Rhizobium huakuii	*Astragalus sinicus*
Bradyrhizobium species:	
Bradyrhizobium japonicum	*Glycine max*
Bradyrhizobium elkanii	*Glycine max*
Azorhizobium species:	
Azorhizobium caulinodans	*Sesbania rostrata*

zobia lack symbiotic plasmids, but have a genetic structure and diversity similar to the population of symbiotic rhizobia (Segovia et al., 1991). This would indicate that sym plasmid loss and gain is a continuous and dynamic process in rhizobia. Furthermore, the acquisition of genetic information to become a pathogen or a symbiont seems to be a very recent event for some lineages of the *Agrobacterium* - *Rhizobium* cluster as discussed before.

R. leguminosarum bv. *phaseoli* seems to be the result of plasmid transfer in historic times. *P. vulgaris* is native to the Americas (Gepts, 1990), and was only introduced into Europe in the XVI century. There are no indigenous species of *Phaseolus* in Europe. Segovia et al. (1993) suggest that *R. etli* strains were introduced with beans to Europe at the same time as their host. Some strains remained as such, but in others sym plasmid transfers occurred into other rhizobia having different chromosomal DNA.

RFLP analysis of *R. galegae* has also shown evidence of plasmid transfer within two geographically distant populations of *R. galegae*. *R. galegae* nodulates two species of goat's rue, *Galega officinalis* and *G. orientalis*, with patterns of *nod-* and *nif* (nitrogen-fixing) genes linked to host-plant specificity. A different grouping was obtained when chromosomal probes were analyzed, some strains of *R. galegae* from *G. officinalis* being more closely related to strains isolated from *G. orientalis* (Kaijalainen and Lindström, 1989). Interstrain transfer of symbiotic sequences in the course of evolution is the most plausible explanation for this.

Rhizobium and *Agrobacterium* strains readily interchange plasmids under laboratory conditions. Different *Rhizobium* species containing Ti (tumor-inducing) plasmids from *Agrobacterium tumefaciens* are tumor-inducing, though the tumors formed are smaller in size (Hooykaas et al., 1977). *Agrobacterium tumefaciens* with symbiotic plasmids from *Rhizobium* form nodules on the corresponding host legume (Hooykaas et al., 1982, 1985; Kondorosi et al., 1982; Truchet et al., 1984; Van Brussel et al., 1982). When *R.*

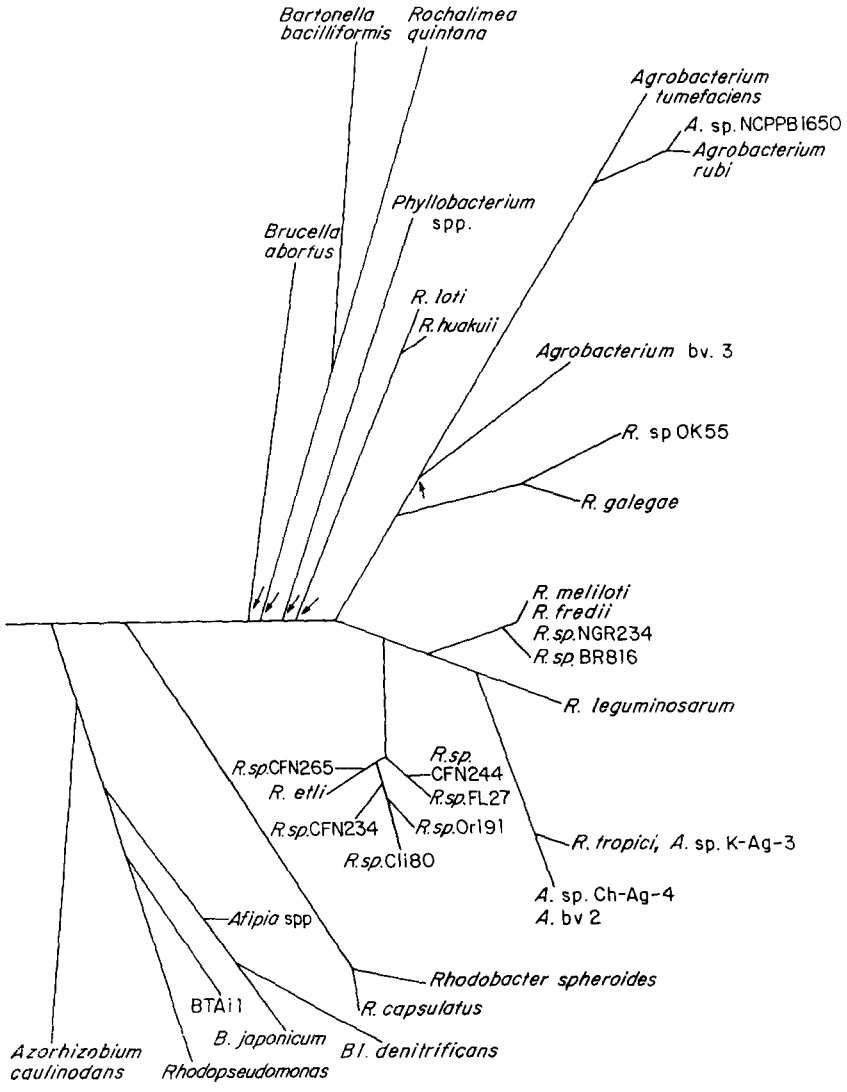

Fig. 1. Phylogenetic tree derived from results obtained by Willems and Collins, 1993; Yanagi and Yamasato, 1993; Sawada et al., 1993; Young et al., 1991 and Hernandez Lucas et al., unpublished. Genetic distances were used to construct the tree by Neighbor-Joining method (Saitou and Nei, 1987). Position of nodes indicated with arrows is not definitive.

tropici sym plasmid was transferred to *A. tumefaciens* plasmid-less strain GMI9023, *A. tumefaciens* transconjugants nodulated and fixed nitrogen in bean, albeit at a reduced level (Martínez et al., 1987). The transconjugants also nodulated *Leucaena* (Fig. 2). As mentioned above, *R. tropici's* closest relatives are *Agrobacterium* spp.

It would be interesting to have more information about *Phyllobacterium* in regard to the existence of plasmids and sequences of putative *nod* genes.

An evolutionary hypothesis

It has been suggested that *Rhizobium* and *Bradyrhizobium* lineages diverged before the origin of legumes (Ochman and Wilson, 1987), and that subsequently, the information required for nodule formation was passed from one genus to the other (Young, 1993). I will present some facts and ideas suggesting that the information flow was from *Bradyrhizobium* to *Rhizobium*. A different hypothesis has been proposed by Janet Sprent (this volume). *Bradyrhizobium* species in general

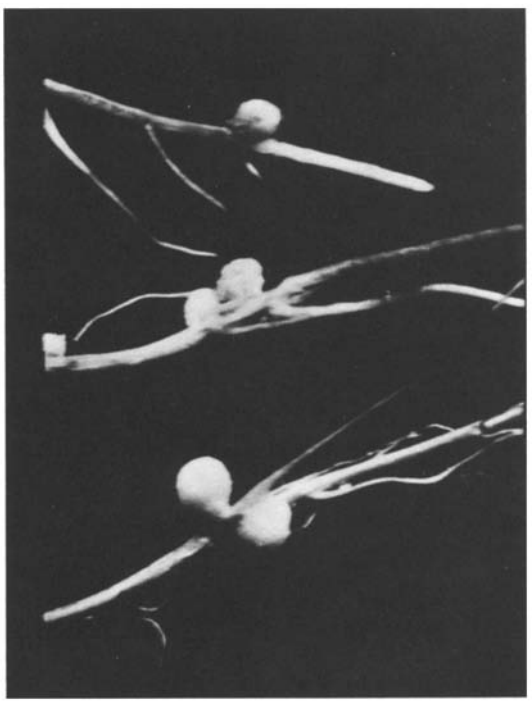

Fig. 2. *Leucaena esculenta* nodules induced by *R. tropici* CFN299 (bottom), *Agrobacterium tumefaciens* GMI9023 transconjugants harboring: pSym and plasmid b from CFN299 (middle) and psym fromCFN299 (up)

have a broader host-range than *Rhizobium*, leading Norris (1956) to propose that *Bradyrhizobium* was the more primitive symbiont. Symbiotic information for nodule formation in legumes could have been transferred from *Bradyrhizobium* to a proto-*Agrobacterium radiobacter,* then after this "catastrophic" event, further distributed with the *Agrobacterium-Rhizobium* chromosomal lineages. Transfer and recombination of symbiotic information could have been the basis for an accelerated evolution that led to *Rhizobium* speciation in relation to legume specificity.

Azorhizobium caulinodans is perhaps more related to *Bradyrhizobium* than to *Rhizobium* by the analysis of 16S ribosomal RNA-genes (Sawada et al., 1993; Willems and Collins, 1993) but *nod*-gene structural similarity is higher between *Bradyrhizobium* and *Rhizobium* than between *Azorhizobium* and *Bradyrhizobium* (Goethals et al., 1989), *nod*-gene information could have originated in an ancestor of the *Azorhizobium* - *Bradyrhizobium* branch, and then diverged in both chromosome in one strain may be plasmid borne in another (Haugland and Verma, 1981). Interestingly pJP4 and r68.45 can be transferred between populations of *Bradyrhizobium* in nonsterile soil with transfer frequencies higher than previously reported for in vitro transfer (Kinkle et al., 1993). It is worthy of mention that repetitive sequences have been found close to gene regions containing the symbiotic information in *B. japonicum*, and this may promote some instability (Hahn and Hennecke, 1988).

Other markers and their linkage in the genome

It is agreed that there is not extensive recombination between *Rhizobium* chromosomes; thus bacteria behave as clones, with linkage between different genetic markers (Piñero et al., 1988; Souza et al., 1992). Thus, it is only necessary to screen specific gene regions to obtain a good image of the whole genome. GSII seems to be a good marker of groups or species in the Rhizobiaceae. *R. meliloti* strains, *R. etli*, *R. tropici* types A and B, *R.leguminosarum* strains have been analyzed in western blots and the isoelectric point of GSII has been determined (Taboada et al., 1993). Bacteria are correctly classified by these means, indicating most probably a common ancestor for each group. Similarly, analysis of fatty acid profiles allows an adequate grouping of rhizobia (Jarvis and Tighe, 1994).

REP- and ERIC-PCR techniques are also useful tools for *Rhizobium* classification. De Bruijn (1992) showed that results from REP-PCR and ERICS-PCR are in agreement with phylogenies derived from multilocus enzyme electrophoresis. Classification of genetically related strains of *Bradyrhizobium japonicum* serocluster 123 by the patterns of their repetitive sequences was correlated with RFLP's (Judd et al., 1993). However repetitive DNA may change faster than the genome as a whole, as it seems to be involved in recombination and amplification events (Flores et

al., 1988). Otherwise, REP-PCR and ERIC-PCR are advantageous because they allow recognition of closely related strains and they are easy and fast to perform.

RFLP analysis of ribosomal genes or PCR-fragments of ribosomal genes are useful to distinguish groups. *Bradyrhizobium* specific probes, *Rhizobium* and *Bradyrhizobium* species-specific and even strain specific probes are starting to be developed (Bjourson et al., 1992; Ludwig, pers. commun.; Wheatcroft and Watson, 1988).

Other DNA sequences

A better sample of the genome would always be more convenient, and this undoubtedly will come in the future, as DNA sequencing becomes more routinely used. In *Salmonella*, it has been found that trees derived from a single gene are not always enough to describe phylogenies (Nelson et al., 1991).

23S rRNA are larger molecules than 16S rRNA, they contain more genetic information that may be useful in phylogenetic analysis (Ludwig et al., 1992). 23S rRNA gene sequences of *Bradyrhizobium* and *Rhodopseudomonas* have been analyzed (Ludwig, personal communication), it would be interesting to have more 23S rRNA sequences from other *Rhizobium* spp.

Phylogenetic trees derived from citrate synthase gene sequence are in general agreement to phylogenies derived from ribosomal genes (Pardo et al., 1994). More sequences of citrate synthase gene from different *Rhizobium* species would be required to draw a complete scheme. *nif* gene phylogeny in *Rhizobium* is linked to the chromosome (Hennecke et al., 1985; Young, 1992).

DNA-DNA homology

Nucleic acid hybridization is considered a reliable means of establishing the relationship between bacterial species, though not of sufficient accuracy. Classically, genomic species encompass strains with approximately 70% or greater DNA relatedness, although the exact level below which organisms are considered to belong to different species varies.

Total DNA-homology as revealed by DNA-DNA hybridization seems not to be in close agreement with 16S ribosomal sequence phylogeny in some cases. This is evident in Table 2 which shows DNA-DNA hybridization results for some of the rhizobia depicted in Fig. 1. DNA-DNA hybridization experiments take into account DNA borne on plasmids. In some *Rhizobium* species, (e.g. *R. etli*) this may represent up to 45% of the genome. Since this extrachromosomal DNA most probably undergoes change faster than core chromosomal DNA, it can contribute to values in DNA homology which are not in clear agreement with other criteria for estimating strain relatedness. We suppose this is, in part, the explanation for the discrepancies between Table 2 and Fig. 1, and for the low DNA:DNA hybridization values reported here.

Bacterial taxonomy on trial

Claims to revise the genus *Agrobacterium* in view of its close relationships to *Rhizobium* have been raised (Sawada et al., 1993; Willems and Collins, 1993). While new species are being proposed, clouds of related rhizobia are starting to emerge, raising questions on realistic limits between species. For example, according to 16S ribsomal RNA partial sequences (Eardly et al., 1992, Hernández-Lucas et al., unpublished; Laguerre et al., 1993) *R.etli* is a branch among other rhizobia with different specificities (Fig. 1). *R. etli* differs from these *R.* spp. in many plasmid-borne traits. We have discussed previously that *R. tropici* is overlapped with *Agrobacterium* spp. In *R. meliloti*, the existence of two highly differentiated evolutionary lineages has been shown. One is adapted to annual medic species of the Mediterranean basin. The genetic distance is so large that it could warrant different species. The extensive genotypic diversity among strains of *R. meliloti* is associated with the unusually high level of species

Table 2. Relative levels of homology at 65°C between the DNA from selected *Rhizobium* species

	Hybridization[a] %
Between *R. tropici* type A strains (average)	91.7%
Between *R. tropici* type B strains (average)	81.4%
R. tropici type A with *R. tropici* type B	39%/36%[b]
R. tropici type A with *Agrobacterium* sp Ch-Ag-4	24%
Between *R. etli* strains (average)	70%
R. etli and *R.*spp. related to *R. etli*[c]	28.3%
R. etli^T CFN42 with *R. leguminosarum* bv. *viciae*	48%/45%[b]
R. etli^T CFN42 with *R. leguminosarum* bv. *trifolii*	49%

[a]Average estimated from Martínez-Romero et al., 1991; Segovia et al., 1991; Martínez et al., unpublished.
[b]Independent result obtained by a different hybridization method by Laguerre and Amarger, INRA, 17 Rue Sully BP 1540, 21034 Dijon-Cédex- France.
[c]R. spp. related to *R. etli*: CFN234, CFN244, CFN265, Cli80 and FL27.

diversity in the genus *Medicago* (Eardly et al., 1990).

While there seems to be an agreement that a biological meaningful classification of *Rhizobium* should be based on chromosomal genes rather than on plasmid-encoded symbiotic characteristics (Young et al., 1993), it seems that bacterial taxonomy has to be deeply changed. We are perhaps waiting for a comprehensive view of the genomes and a more complete scope of existing microorganisms to set the rules, but changing seems difficult. The true impact of taxonomy would be not only to give names but to provide a true conceptual framework for research. The concept of genetic isolation is certainly not true in bacterial species, and different species sharing plasmids would be perhaps not uncommon.

The known microorganisms are only a very small proportion of existing organisms (Torsvik et al., 1990). This is specially true for *Rhizobium* and *Bradyrhizobium* where only a small number of nodulating legumes have been sampled for their symbionts. It has been estimated that at least 2800 species of legumes form nodules (Allen and Allen, 1981), yet the 8 *Rhizobium* species and two *Bradyrhizobium* species listed in Table 1 represent less than 1% of the nodulated species of legumes. A number of tree and tropical legumes may be nodulated by both *Bradyrhizobium* or *Rhizobium* spp. (Martínez et al., 1985; Zhang et al., 1991). Very convenient schemes to characterize and classify such rhizobia have been proposed (Graham et al., 1991) and need to be followed up.

Bacterial diversity is perhaps the most valuable resource for biotechnology. Biologists have only begun to assess the complexity and potentiality of each bacterial species (Bull et al., 1992).

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