Molecular genetics of *Azorhizobium phenotypic* and genotypic studies on tropical rhizobia leading to the characterization of *Azorhizobium caulinodans*

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I. Introduction

The genus *Rhizobium* (which means etymologically that which lives in the roots') was created in 1889 (7) for those bacteria which nodulate the roots of leguminous plants, wherein these bacteria live as endosymbionts.

Since then, their classification has been changed considerably from a system based mainly on plant cross inoculation groups (8,13) to a scheme based on results involving large parts if not total bacterial genomes (2, 9, 10). In the first edition of Bergey's Manual of Systematic Bacteriology, Jordan (14) distinguishes within the rhizobia two genera, *Rhizobium* and *Bradyrhizobium* with respectively 3 and 1 species. The comparison of ribosomal RRNA'S by cataloguing (18) or DNA-rRNA hybridizations (3, 6, 10) has proven to be an excellent tool to study intergeneric and even more remote relationships of bacteria. DNA-rRNA hybridizations have been used to unravel in more detail the inter- and intrageneric relationships with and within *Rhizobium* and *Bradyrhizobium* (10). Both genera belong in rRNA superfamily IV, but are further removed from each other than they are from other genera in this rRNA superfamily. *Rhizobium* is more closely related to *Agrobacterium*; *Bradyrhizobium* to *Rhodopseudomonas palustris* and *Nitrobacter*. *R. loti* occupies a separate position and the taxonomic position of *R. fredii* and rhizobia isolated from *Galega* species has been determined. One stemnodulating *Sesbania* strain was preliminary included in this study (10): it appeared to occupy a separate position in rRNA superfamily IV.

The aim of our work was to determine the exact taxonomic structure and status of the stem-nodulating *Sesbania* strains and to reveal their closest relatives by a polyphasic approach, involving different modern methods allowing differentiation on different taxonomic levels. The methods used were: numerical taxonomy of phenotypic features, comparison of the SDS gelelectrophoretic wholecell protein patterns, total DNA-DNA hybridizations, DNA-rRNA hybridizations and determination of their % (G+C).

The results lead to the proposal of a new genus for the *Sesbania* root- and stem- nodulating bacteria *Azorhizobium*; this new genus contains one species *Azorhizobium caulinodans*.

II. Phenotypic results

A total of 20 strains isolated from stem nodules of *S. rostrata* were compared by methods of numerical taxonomy with 9 fast growing rhizobia strains isolated from root nodules of different *Sesbania* species, with 20 other strains of the genus *Rhizobium* and with 17 strains of the genes *Bradyrhizobium*. The latter groups contained both own isolates from different *Acacia* species and from *Leucaena leucocephala* from Senegal. We compared 221 characters including 151 sole carbon sources. The results of the complete linkage cluster analysis (Figure 1) reveal three clusters corresponding to *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, which constitutes a homogeneous phenon, distinct from the other two, although a bit closes to the latter.

The fast growing root-nodulating strains from tropical *Acacia* and *Leucaena* species belong together with the *Sesbania* root-nodulating strains in the *Rhizobium* cluster in which we distinguish 4 subphena. Further studies with more tropical rhizobia (unpublished results from K. Kersters and B. Dreyfus) confirm and even extend this heterogeneity since more strains were found in the 4 subphena but also new subphena were detected. The *Bradyrhizobium* cluster contains 2 subphena. The features differentiating the stem-nodulating strains (*Azorhizobium*) from *Rhizobium* and *Bradyrhizobium* were determined. The most striking presence of one lateral flagellum when grown in liquid medium, their colony morphology, their generation time, the lack of sugar assimilation (except glucose). Moreover the *Sesbania* stem-nodulating bacteria con fix N_2 in culture under microaerobic conditions and grow at the expense of this fixed N_2 (4). This seems to be (together with the stem-nodulating capacity) an important discriminative character between *Azorhizobium*, *Rhizobium* and *Bradyrhizobium*, although some strains belonging to *Rhizobium* have been described as showing some degree of ex-planta nitrogen-fixing ability (1, 15). When compared with the stem-nodulating *Sesbania* strains the nitrogenase activity and oxygen tolerance are the highest for the latter strains (30 nmol of C_2H_2 produced per mg of protein per min, and an oxygen tolerance up to 9 nmol).

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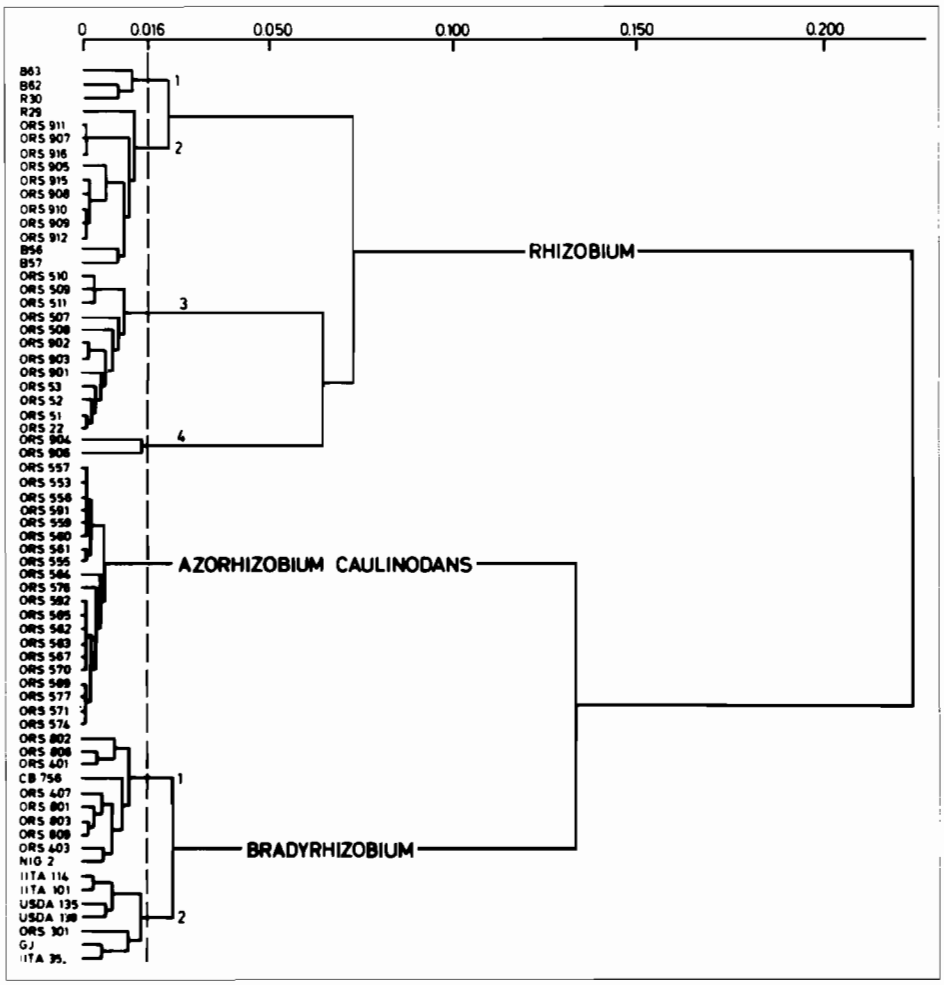

Fig. 1. Dendrogram showing the results of the complete linkage cluster analysis of phenotypic features.

All strains from the *Azorhizobium* cluster formed effective stem and root nodules. Among the *Sesbania* root-nodulating *Rhizobium* strains, 2 strains formed pseudonodules on the stems of *Sesbania rostrata*. Two strains (ORS 609 and ORS 611), isolated from root nodules of *S. cannabina* and *S. grandiflora* were capable to form effective nodules on both roots and stems, but did not fix N_2 in culture. Both strains were not included in the phenotypic analysis but our genotypic results indicated that they were authentic rhizobia.

III. DNA:rRNA hybridization results

Initially, we hybridized DNA from representative strains belonging to the *Rhizobium* phenon (ORS 52 and ORS 22) and the *Sesbania* stem-nodulating phenon (ORS 571) with 3 appropriate rRNA probes available in our research group. The results (10) located ORS 22 and ORS 52 in the *Rhizobium-Agrobacterium* rRNA complex in rRNA superfamily IV. Strains ORS 571 was located at the bifurcation level (70.5 C) of the *Bradyrhizobium-Rhodopseudomonas palustris* rRNA branch and the *Beijerinckia* rRNA branch. Theoretically the other taxa from this $T_{m(e)}$ level can be more closely related to strain ORS 571: *Methylobacterium, Xanthobacter, Rhodopseudomonas acidophila* and *Rhodopseudomonas viridis*. In order to unravel these relationships, we prepared a [^{3}H]-labelled rRNA probe from strain ORS 571 and hybridized it with DNA's from other members of the stem-nodulating *Sesbania* phenon and with DNA's from the above mentioned possibly related bacteria.

The results are shown in a $T_{m(e)}$ dendrogram (Figure 2), representing part of the rRNA superfamily IV.

The most useful and significant parameter of a DNA:rRNA hybrid is its $T_{m(e)}$-value (3, 6, 10, 17), because this parameter is a measure of the base sequence similarity between rRNA cistrons and has a decisive taxonomic significance. Our results show that the *Sesbania* stem-nodulating bacteria constitute a separate rRNA subbranch on the *Rhodopseudomonas palustris-Bradyrhizobium* rRNA branch. Four representative strains constitute a very narrow cluster ($T_{m(e)}$ from 80.8 to 81.6 C) belongs also on this subbranch, and this genus is thus the closest relative of the *Sesbania* stem-nodulating strains.

These strains cannot be included in *Xanthobacter* because a difference in $T_{m(e)}$ of 4 C can indeed reflect an intergeneric relationship, provided that sufficient phenotypic arguments are available to differentiate both genera. Since this condition was indeed fulfilled we consequently proposed a separate genus rank for the *Sesbania* stem-nodulating strains. At the moment of this conclusion only 2 species were described in *Xanthobacter* (16) and we made our differentiating table [Table 4 in (5)] according to the available data. Recently (11), a third species *X. agilis* has been described and *X. flavus* was emended (12). The type strain of *X. agilis* has since then also been hybridized with the [^{3}H]-rRNA probe from strain ORS 571, it has a $T_{m(e)}$ value of 77 C, indicating that it is also a member of the *Xanthobacter* rRNA cluster. When the phenotypic results of the revised genus *Xanthobacter* were compared with these of the *Sesbania* stem-nodulating cluster, we still found enough features to differentiate both genera. The revised differentiating table will be published elsewhere (M. Gillis, J.L. Garcia and B. Dreyfus, manuscript in preparation).

Strains ORS 609 and ORS 611 have $T_{m(e)}$ values of 79.7 C versus the rRNA probe from *R. meliloti* NZP 4009 showing that they are members of the *Rhizobium-Agrobacterium* rRNA complex.

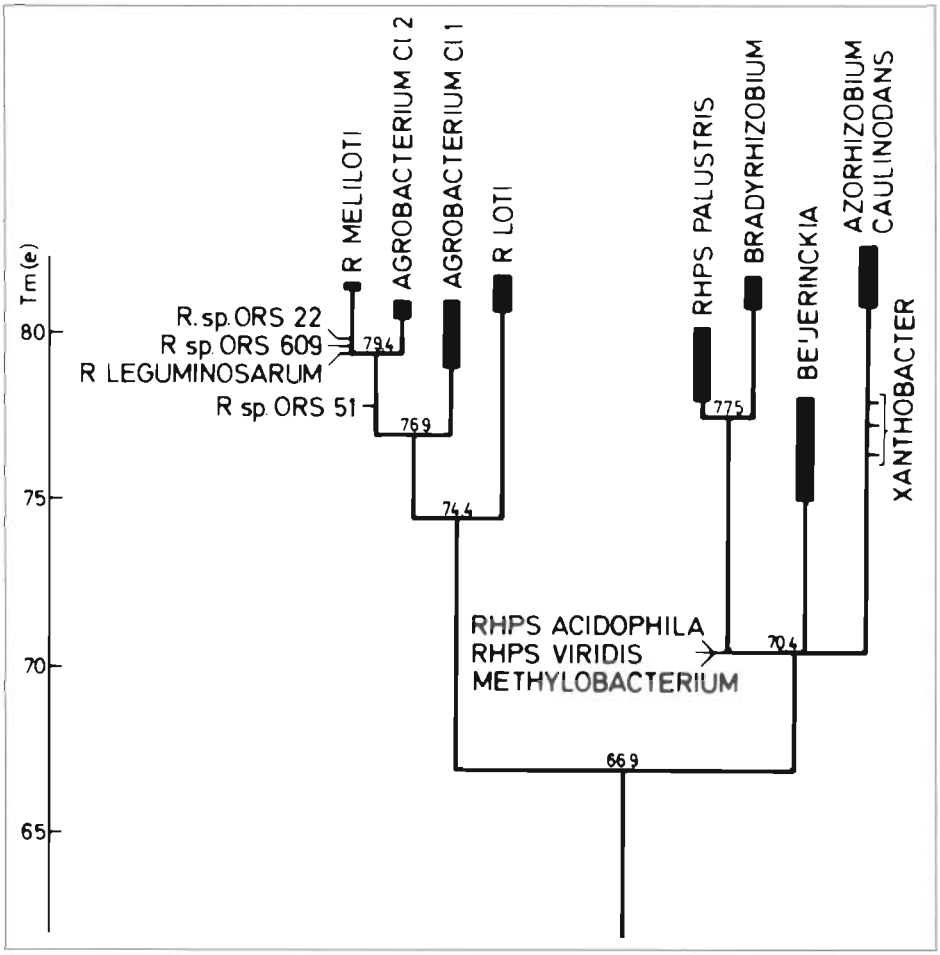

Fig. 2. Simplified $T_{m(e)}$ cistron similarity dendrogram of part of rRNA superfamily IV.

IV. Comparative sds gel-electrophoresis of whole-cell proteins and DNA-DNA hybridizations

Four strains of the stem-nodulating *Sesbania* strains have almost identical protein electrophoregrams (Figure 3) indicating that they constitute indeed a very homogeneous cluster. High percentages of total DNA:DNA binding (95%) were indeed found between representative strains of the *Sesbania* stem-nodulating bacteria showing that these strains belong genotypically in one species.

V. Conclusions

- 1.Tropical rhizobia are heterogeneous and more phenotypic and genotypic research is necessary to unravel their relationships.
- 2.We propose a new genus and new species for the stem-nodulating *Sesbania* strains because the DNA:rRNA hybridization results show clearly that these strains constitute a separate rRNA subbranch and do not belong in *Rhizobium* nor in *Bradyrhizobium*. *Xanthobacter* (3 species) appears to be their closest relative, from which they are phenotypically sufficiently different to deserve a separate generic rank.
- 3.Because the capability of fixing high amounts of N_2 and growing at the expense of this fixed N_2, while free living, is a quite discriminative feature of the new genus we proposed to name it *Azorhizobium*.
- 4.Within this genus we found genotypically and phenotypically only one species, which we named *caulinodans* according to its stem-nodulating capacity.
  The type strain is *Azorhizobium caulinodans* ORS 571 which was deposited in the collection of the Laboratory of Microbiology in Gent (LMG 6465). The G+C content of the DNA is 66.5 mol %. The complete description of the new genus and species can be found in (5).

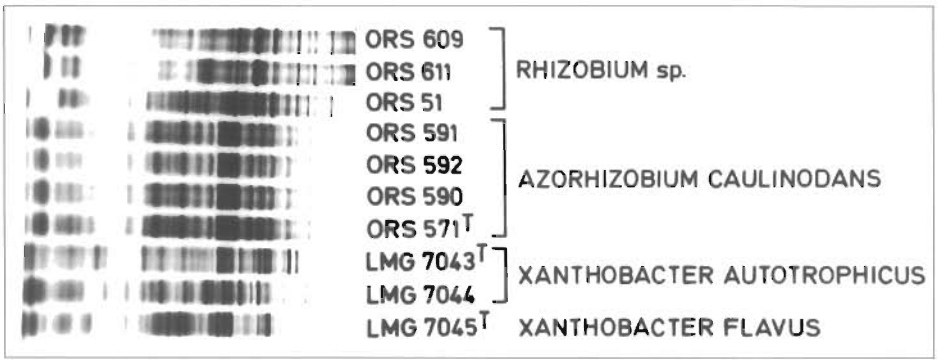

Fig. 3. Normalized SDS-polyacrylamide gel electrophoretic patterns of four Azorhizobium caulinodans strains, three Rhizobium sp. and three Xanthobacter strains.

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