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Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a Stem-Nodulating Nitrogen-Fixing Bacterium Isolated from *Sesbania rostrata*

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Twenty stem- and root-nodulating bacterial strains isolated from stem nodules of *Sesbania rostrata* were compared by numerical analysis of 221 phenotypic features with nine strains which effectively nodulate only the roots of this plant and with representative strains from the genera *Rhizobium* and *Bradyrhizobium*. Representative organisms from the different clusters were investigated further, together with possibly related organisms, by performing comparative gel electrophoresis of whole-cell proteins and by performing deoxyribonucleic acid (DNA)-DNA and DNA-ribosomal ribonucleic acid (rRNA) hybridizations. ^{3}H-labeled rRNA was prepared from *Sesbania* stem- and root-nodulating bacterial strain ORS 571^T (T = type strain); [^{14}C]rRNA from *Bradyrhizobium japonicum* NZP 5549^T was also used. The following conclusions were drawn: (i) the *Sesbania* root-nodulating bacterial strains are genuine rhizobia; (ii) the *Sesbania* stem- and root-nodulating strains are quite different from *Rhizobium* and *Bradyrhizobium*, and thus they constitute a separate rRNA subbranch on the *Rhodopseudomonas palustris* rRNA branch in rRNA superfamily IV; and (iii) the closest relative of these organisms is *Xanthobacter*, but they are phenotypically and genotypically sufficiently different from the latter genus to deserve a separate generic rank. Because the feature of free-living nitrogen fixation is quite discriminative, a new genus, *Azorhizobium*, is proposed, with one species, *Azorhizobium caulinodans*. The type strain is strain ORS 571 (= LMG 6465).

Bacteria which form nitrogen-fixing nodules on leguminous plants are currently divided into two genera, *Rhizobium* and *Bradyrhizobium* (17). The genus *Rhizobium* comprises four species, *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Rhizobium loti*, and *Rhizobium fredii*. The species *Rhizobium leguminosarum* regroups three former species, *Rhizobium trifolii*, *Rhizobium phaseoli*, and *Rhizobium leguminosarum*. All species of the genus *Rhizobium* are fast-growing bacteria. The genus *Bradyrhizobium* comprises one well-defined species, *Bradyrhizobium japonicum*, and includes all of the bacteria previously referred to as slow-growing rhizobia.

Nitrogen-fixing nodules are usually found on the roots of the host leguminous plants. Only some legume species belonging to the genera *Neptunia*, *Aeschynomene*, and *Sesbania* bear nodules on both roots and stems (9). Recently, Dreyfus et al. (8) found that the tropical legume *Sesbania rostrata* was actually associated with the two types of strains described below. (i) Some strains are similar to strain ORS 571^T (T = type strain), which fixes atmospheric N_2 in culture and grows at the expense of this fixed N_2; they always nodulate both roots and stems of *S. rostrata* (10, 13). They are usually cited in the literature as "the *Sesbania* stem-nodulating strains." We did not find such N_2-fixing strains in stem nodules from *Neptunia* and *Aeschynomene* (B. Dreyfus, unpublished data). (ii) Some strains do not fix N_2 in culture and generally nodulate the roots of *S. rostrata* only.

Recent results based on deoxyribonucleic acid (DNA)-ribosomal ribonucleic acid (rRNA) hybridizations (16) indicate that two representative *Sesbania* root-nodulating strains belong to the genus *Rhizobium*. On the other hand, stem- and root-nodulating strain ORS 571^T has been shown to belong to the *Rhodopseudomonas palustris* rRNA branch and consequently to be a relative of the latter taxon and of *Bradyrhizobium*, although it does not belong in either of these taxa.

To determine the exact taxonomic position of this new group of N_2-fixing, nodulating organisms, we analyzed a collection of root- and stem-nodulating strains from *S. rostrata* from different geographical areas and compared them with *Sesbania* root-nodulating strains and strains of *Rhizobium* and *Bradyrhizobium*. These investigations included a numerical analysis of phenotypic features, comparative protein gel electrophoresis, and DNA-DNA and DNA-rRNA hybridizations.

The results of this work led to a proposal for a new genus for the *S. rostrata* root- and stem-nodulating bacteria, *Azorhizobium*, which we describe here; this genus contains one species, *Azorhizobium caulinodans*. Below we use the latter name.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains which we studied are listed in Table 1. The purity of the cultures was assured by repeated use of single-colony isolates, and the identities of the nodulating strains were checked by plant infection tests on their original host plants. A total of 21 stem- and root-nodulating strains (*Azorhizobium*) were isolated from stem nodules of *S. rostrata* growing in different regions of Senegal and Madagascar by using standard procedures (29). Eleven root-nodulating strains (*Rhizobium*) of *S. rostrata* were originally isolated from root nodules of *S. rostrata*, *Sesbania pachycarpa*, *Sesbania cannabina*, and *Sesbania grandiflora*. Fourteen *Rhizobium* strains were isolated from nodules of *Acacia* sp. and *Leucaena* sp. All of these isolates (except strains ORS 590, ORS 609, and ORS 611) were

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TABLE 1. Strain designations, origins, and comments

Strain(s)[a]	Origin[b]	Received from[a]:	Comments
Azorhizobium caulinodans ORS 571^T (= LMG 6465^T), ORS 553, ORS 555, ORS 556, ORS 557, ORS 559, ORS 560, ORS 561, ORS 562, ORS 563, ORS 564, ORS 565, ORS 567, ORS 569, ORS 570, ORS 574, ORS 576, and ORS 577	*S. rostrata*, from Senegal	Our isolate	Nodulates roots and stems of *S. rostrata*
Azorhizobium caulinodans ORS 590	*S. rostrata*, from Senegal	I. Ndoye	Nodulates roots and stems of *S. rostrata*
Azorhizobium caulinodans ORS 591 and ORS 592	*S. rostrata*, from Madagascar	R. Fettiarisson	Nodulates roots and stems of *S. rostrata*
Rhizobium sp. strain ORS 22 (= LMG 6463)	*S. rostrata*, from Senegal	Our isolate	Nodulates only roots of *S. rostrata*
Rhizobium sp. strain ORS 51 (= LMG 6464)	*S. rostrata*, from Senegal	Our isolate	Nodulates roots and forms pseudonodules on stems of *S. rostrata*
Rhizobium sp. strains ORS 52 and ORS 53	*S. rostrata*, from Senegal	Our isolate	Nodulates only roots of *S. rostrata*
Rhizobium sp. strain ORS 507	*S. pachycarpa*, from Senegal	Our isolate	Nodulates roots and forms pseudonodules on stems of *S. rostrata*
Rhizobium sp. strains ORS 508, ORS 509, ORS 510, and ORS 511	*S. pachycarpa*, from Senegal	Our isolate	Nodulates only roots of *S. rostrata*
Rhizobium sp. strain ORS 609	*S. cannabina*, from Senegal	I. Ndoye	Nodulates roots and stems of *S. rostrata*
Rhizobium sp. strain ORS 611	*S. grandiflora*, from Senegal	Our isolate	Nodulates roots and stems of *S. rostrata*
Rhizobium sp. strains ORS 901, ORS 902, ORS 903, ORS 906, and ORS 909	*Acacia senegal*, from Senegal	Our isolate	
Rhizobium sp. strains ORS 904, ORS 905, ORS 915, and ORS 916	*Leucaena leucocephala*, from Senegal	Our isolate	
Rhizobium sp. strain ORS 907	*Acacia raddiana*, from Senegal	Our isolate	
Rhizobium sp. strains ORS 908 and ORS 910	*Acacia bivenosa*, from Senegal	Our isolate	
Rhizobium sp. strains ORS 911 and ORS 912	*Acacia farnesiana*, from Senegal	Our isolate	
Rhizobium meliloti B56, B57, and R29	*Medicago sativa*, from United States	E. L. Schmidt	
Rhizobium meliloti R30	*Medicago sativa*, from Colombia	CIAT	
Rhizobium meliloti LMG 6130 (= NZP 4009)	*Medicago sativa*, from New Zealand	B. D. W. Jarvis	
Rhizobium leguminosarum B62 and B63	*Pisum sativum*, from United States	E. L. Schmidt	
Bradyrhizobium japonicum USDA 135 and USDA 138	*Glycine max*, from United States	USDA	
Bradyrhizobium japonicum IITA 114, IITA 101, and IITA 35	*Glycine max*, from Nigeria	IITA	
Bradyrhizobium japonicum LMG 6138^T (= NZP 5549^T)	*Glycine max*, from New Zealand	B. D. W. Jarvis	
Bradyrhizobium sp. strains NZP 2314 and NZP 2309	*Lotus pendiculatus*, from New Zealand	B. D. W. Jarvis	
Bradyrhizobium sp. strain ORS 801	*Acacia holosericea*, from Senegal	Our isolate	
Bradyrhizobium sp. strain ORS 802	*Acacia sieberiana*, from Senegal	Our isolate	
Bradyrhizobium sp. strain ORS 803	*Acacia bivenosa*, from Senegal	Our isolate	
Bradyrhizobium sp. strain ORS 806	*Leucaena leucocephala*, from Senegal	Our isolate	
Bradyrhizobium sp. strain ORS 809	*Acacia albida*, from Senegal	Our isolate	
Bradyrhizobium sp. strains ORS 401, ORS 403, and ORS 407	*Glycine max* cv. Malayan, from Senegal	Our isolate	
Bradyrhizobium sp. strain NIG 2	*Glycine max* cv. Malayan, from Senegal	IITA	
Bradyrhizobium sp. strain GJ	*Glycine javanica*, from Senegal	Our isolate	
Bradyrhizobium sp. strain ORS 301	*Aeschynomene afraspera*, from Senegal	Our isolate	
Bradyrhizobium sp. strain CB 756	*Macrotylama africanum*, from Zimbabwe	CSIRO	
Rhodopseudomonas viridis DSM 135		DSM	
Rhodopseudomonas acidophila GD14		N. Pfennig	
Rhodopseudomonas acidophila ATCC 25092^T		ATCC	
Xanthobacter autotrophicus LMG 7043^T (= DSM 432^T) and LMG 7044 (= DSM 1393)		DSM	
Xanthobacter flavus LMG 7045^T (= DSM 338^T)		DSM	
Beijerinckia indica NCIB 8712^T (= LMG 2817^T)		NCIB	
Methylobacterium radiotolerans IAM 12099		Y. Nishimura	
Methylobacterium sp. strain 317 (= LMG 6085)		P. Green	

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compared with four strains of *Rhizobium meliloti*, two strains of *Rhizobium leguminosarum*, and 17 strains of *Bradyrhizobium japonicum* and *Bradyrhizobium* sp. (cowpea strains) by using numerical taxonomy of phenotypic features. Representative strains from the different clusters and from the *Rhodopseudomonas palustris* and *Beijerinckia* rRNA branch (16) were investigated further by using DNA-DNA hybridization or DNA-rRNA hybridization or both and comparative gel electrophoresis of the cellular proteins.

Growth media. All cultures of *Rhizobium* and *Bradyrhizobium* were grown and maintained on yeast extract-mannitol (YM) medium with or without agar (29). Strains of *Azorhizobium* were grown on lactate medium YL (10) or YM liquid medium. For the phenotypic tests, the strains were grown on medium containing 1.67 g of K_2HPO_4, 0.87 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.05 g of NaCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 40 mg of $CaCl_2$, 4 mg of $FeCl_3$, 15 g of Noble agar (Difco Laboratories), 1 to 2 g of carbon source, 1 ml of a trace element solution (10), 2 ml of a vitamin solution (10), and distilled water to a volume of 1,000 ml, pH 6.8. For nitrogen fixation in culture, nitrogen-free medium LO was used (10) under microaerobic conditions (3% O_2, 97% N_2).

Rhodopseudomonas palustris, *Beijerinckia indica*, and *Xanthobacter* strains were grown as described previously (5, 16). *Rhodopseudomonas viridis* and *Rhodopseudomonas acidophila* were grown anaerobically on medium 27 (1).

Staining methods. Gram staining was done by the method of Burke (7), and flagella were visualized by using the technique of Rhodes (26). Poly-β-hydroxybutyric acid was determined by the method of Juthner et al. (18). For electron microscopy we used preparations negatively stained with uranyl acetate.

Biochemical assays for numerical taxonomy. The relevant characters used in the numerical taxonomy analysis are listed in Table 2. To test for utilization of sole carbon sources, the medium described above was used. A total of 151 organic substrates were added after filter sterilization (pore size, 0.45 μm; Millipore Corp.) to the medium at concentrations of 2 g/liter for sugars and 1 g/liter for alcohols, organic acids, amino acids, and other compounds except phenol; phenol was used at a concentration of 0.25 g/liter. Geraniol and naphthalene were added in the inverted covers of petri dishes after inoculation. Inoculation was performed with a multipoint inoculator (22), and duplicate petri dishes were incubated at 30°C for 4 to 10 days. Growth was considered positive when there was a difference in growth compared with a control plate without carbon substrate. Physiological tests, such as tests for oxidase, catalase, urease, hydrolysis of gelatin, egg yolk, or starch, and oxidation-fermentation, were performed by the methods described in the *Manual of Methods for General Bacteriology* (14). The other diagnostic characters used were determined as described by Garcia et al. (12).

Plant infection tests. Seeds of *S. rostrata* were surface sterilized with concentrated H_2SO_4 (30 min), washed, and germinated on soft agar. Seedlings were planted in cotton-plugged test tubes containing Jensen seedling agar (29). The stems of *S. rostrata* were inoculated by spraying them with liquid cultures of the test strains.

Nitrogenase assays. Preliminary tests were performed in 10-ml rubber-capped tubes containing 2 ml of semisolid LO medium and 3% O_2–2% C_2H_2–95% N_2 gas phase. Samples of gas were analyzed after 24 h for C_2H_4 production by using a flame ionization gas chromatograph (Varian Aerograph 1400). Strains with positive nitrogenase activity were then grown in YM or YL liquid medium; after centrifugation and resuspension, the cells were incubated in LO medium under an atmosphere containing 3% O_2 and 97% N_2 for growth on N_2 (10).

Computer analysis. Characters were coded 1 for positive and 0 for negative. The final $n \times t$ matrix contained 67 strains and 221 characters. After calculation of similarities among strains, clusters were formed by using the complete-linkage clustering method. The distance between strains and groups was calculated by using the chi-square (χ^2) criterion (11). The data were then plotted as a dendrogram. The analysis was performed with a PDP II computer at the Laboratoire de Microbiologie du Milieu Marin, Université de Provence, Marseille, France.

DNA-rRNA hybridizations. DNA was prepared as described previously (5, 16). For fixation of high-molecular-weight, single-stranded DNA on filters, we used the procedure described by De Ley and Tytgat (3) and Sartorius type SM 11358 membrane filters. [^{3}H]rRNA from *Azorhizobium caulinodans* ORS 571^T was prepared as described by Van Landschoot and De Ley (28). The growth medium used was medium A (5) supplemented with 2% glucose and 1 mCi of [6-^{3}H]orotate.

DNA-rRNA hybridizations were carried out as described previously (5, 6, 16). The following two parameters were measured: (i) $T_{m(e)}$, which was the temperature at which 50% of the hybrid was denatured; and (ii) the percentage of labeled rRNA duplexed (micrograms per 100 μg of DNA fixed on the filter after ribonuclease treatment). Both parameters were derived from the melting curve of the hybrid. After simulation of the hybridization step as described by De Smedt and De Ley (5), each concentration of DNA was determined by the method of Richards (27).

DNA base composition. The average guanine-plus-cytosine (G+C) contents of the DNAs from the strains investigated were measured by the thermal denaturation method (4) and were calculated by using the equation of Marmur and Doty (23), as modified by De Ley (2).

Polyacrylamide gel electrophoresis of total cellular proteins. Protein samples were prepared from whole cells (grown in two Roux flasks on YEB medium containing 0.5% [wt/vol] peptone, 0.1% [wt/vol] yeast extract, 0.5% [wt/vol] beef extract, and 0.5% [wt/vol] sucrose) by treating the cells with sodium dodecyl sulfate as described previously (20). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was per-

[a] ATCC, American Type Culture Collection, Rockville, Md.; CIAT, Centro International de Agricultura Tropical, Cali, Colombia; CSIRO, Commonwealth Scientific and Industrial Research Organisation, Homebush, New South Wales, Australia; CIP, Collection de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; IITA, International Institute for Tropical Agriculture, Ibadan, Nigeria; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, Gent, Belgium; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; NZP, Culture Collection of the Department for Scientific and Industrial Research, Applied Biochemistry Division, Palmerston North, New Zealand; ORS, Collection de l'O.R.S.T.O.M., Dakar, Senegal; USDA, U.S. Department of Agriculture, Beltsville, Md.; R. Fettiarisson, FOFIFA, Antananarivo, Madagascar; P. Green, Torry Research Station, Aberdeen, Scotland; B. D. W. Jarvis, Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand; I. Ndoye, O.R.S.T.O.M., Dakar, Senegal; Y. Nishimura, Department of Applied Biological Science, Science University of Tokyo, Noda, Japan; N. Pfennig, Universität Konstanz, Konstanz, Federal Republic of Germany; E. L. Schmidt, University of Minnesota, St. Paul.

[b] Information was not available for some strains.

TABLE 2. Phenotypic features differentiating *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium*

Feature	*Rhizobium* (29 strains)	*Azorhizobium* (20 strains)	*Bradyrhizobium* (17 strains)
One lateral flagellum (liquid medium)	–[a]	+	–
One subpolar flagellum (solid medium)	–	–	+
Peritrichous flagella (solid medium)	+	+	–
Growth at 12°C	51	+	–
Growth at 44°C	–	+	–
Growth in the presence of:			
0.5% NaCl	79	–	–
8% KNO_3	72	+	–
Growth at pH 8	+	+	–
Acidification on glucose	72	–	–
Alkalinization on glucose	21	+	+
Arginine dihydrolase	13	+	–
Lysine decarboxylase	–	+	–
Denitrification	82	–	41
Growth on:			
D-Ribose, D-xylose, D-mannose, D-galactose, D-arabinose, L-arabinose	+	–	+
D-Maltose, D-cellobiose, lactose, inositol, raffinose	+	–	–
Azelate, maleate, adipate, pimelate, suberate	–	+	+
L-Tartrate, *meso*-tartrate	–	–	+
1,2-Propanediol, 2,3-butanediol, itaconate	–	+	–
L-Xylose	69	–	59
L-Rhamnose	+	–	65
D-Fructose, sucrose, trehalose	+	–	82
Inuline, pelargonate	–	–	53
Gluconate	31	+	+
Mucate, crotonate, nicotinate	–	+	82
α-Methylglycoside, benzoate	34	–	–
2-Ketogluconate	83	+	71
Propionate	52	+	65
Butyrate	–	+	65
Isobutyrate	28	+	76
Valerate	38	+	65
Isovalerate	–	+	71
Caproate	–	+	68
Laurate	–	+	59
Caprate	–	–	24
Oxalate	–	–	29
Malonate	34	+	35
Succinate, 2-ketoglutarate	33	+	+
Fumarate	+	+	59
Glutarate	34	+	82
Sebacate	–	+	88
DL-Malate	79	+	88
DL-Lactate	79	–	41
Citrate	55	+	+
Pyruvate	86	+	+
Aconitate	38	+	29
Levulinate, *p*-hydroxybenzoate	–	–	76
Citraconate	–	+	29
Formate	–	–	18
Tartronate	–	–	12
D-Glucuronate	38	+	35
α-D-Galacturonate	38	+	82
cis-Aconitate	38	+	47
Erythritol	45	–	12
Mannitol	+	–	88
Sorbitol	+	–	35
Ribitol	+	–	76
m-Hydroxybenzoate, L-aspartate	45	+	+
Quinate	69	+	65
L-α-Alanine	83	+	66
β-Alanine, ethanolamine, L-arginine	72	–	–
L-Lysine	76	+	–
γ-Aminobutyrate	79	–	–
L-Histidine	76	–	–
DL-Proline	79	+	24

Continued on following page

TABLE 2—*Continued*

Feature	*Rhizobium* (29 strains)	*Azorhizobium* (20 strains)	*Bradyrhizobium* (17 strains)
L-Phenylalanine	−	−	35
L-Asparagine	66	+	+
Methylamine	24	−	−
D-Glucosamine	79	−	41
Betaine	79	+	−
Sarcosine	13	+	−
Hippurate	38	−	−
Pantothenate	−	−	65
Acetamide	−	25	88

[a] +, 90% or more of the strains positive; −, 90% or more of the strains negative. The numbers are percentages of positive strains.

formed by the method of Laemmli (21), using minor modifications described previously (20).

RESULTS AND DISCUSSION

Phenotypic features. A total of 20 strains isolated from stem nodules (Fig. 1) of *S. rostrata* were compared by using the methods of numerical taxonomy with 9 fast-growing strains isolated from different *Sesbania* species, with 20 strains of the genus *Rhizobium*, and with 17 strains of the genus *Bradyrhizobium*. The numerical taxonomy analysis was performed on 221 characters, including 151 sole carbon sources. The following 29 compounds were utilized by none of the strains as sources of carbon and energy: starch, sucrose, palmitate, D-tartrate, geraniol, *o*-hydroxybenzoate, phthalate, gallate, terephthalate, phenylacetate, *trans*-cinnamate, naphthalene, phenol, testosterone, DL-serine, DL-threonine, DL-α-aminobutyrate, DL-norvaline, L-cystine, kynurenate, L-methionine, benzylamine, spermine, tryptamine, creatine, *n*-dodecane, *n*-hexadecane, DL-ornithine, and glycolate. All of the strains used D-glucose, acetate, DL-glycerate, L-glutamate, and L-leucine. They all formed NO_2^- from NO_3^-. The results of the complete-linkage cluster analysis are shown in Fig. 2. We drew the conclusions described below from these studies.

(i) The 20 root- and stem-nodulating strains from *S. rostrata* (including strain ORS 571^T) form a very distinct and homogenous cluster defined at the 0.005-χ^2 index level. This cluster is distinct from the two other well-defined clusters, the *Rhizobium* cluster and the *Bradyrhizobium* cluster, although it is more closely related to the latter.

(ii) The *Acacia*-nodulating strains analyzed seem to belong to the *Rhizobium* cluster. Within the *Rhizobium* cluster the following four phena can be distinguished at the 0.016-χ^2 index level: (i) three strains from temperate legumes (namely, two *Rhizobium meliloti* strains and one *Rhizobium leguminosarum* strain) constitute phenon 1; (ii) phenon 2 consists of nine rhizobia from *Acacia* sp. and three *Rhizobium meliloti* strains; (iii) all of the *Sesbania* root-nodulating strains included and three strains from *Acacia* constitute phenon 3; and (iv) one strain from *Acacia* and one strain from *Leucaena* constitute phenon 4. Fast-growing tropical rhizobia have not yet been studied thoroughly before (17); although we found that these organisms appear to be closely related to *Rhizobium meliloti* and *Rhizobium leguminosarum*, further study will be necessary to determine their exact taxonomic position. For two representative *Sesbania* root-nodulating strains (ORS 22 and ORS 51), it has also been shown by DNA-rRNA hybridization that these organisms are closely related to *Rhizobium meliloti* and *Rhizobium leguminosarum* (16).

(iii) Within *Bradyrhizobium* we can distinguish at a comparable χ^2 level two phena (phena 2 and 1) corresponding, respectively, to *Bradyrhizobium japonicum* and the *Bradyrhizobium* strains constituting the so-called cowpea miscellany.

(iv) The cluster formed by the *Sesbania* stem-nodulating strains is sufficiently different from *Rhizobium* and *Bradyrhizobium* to suggest the possibility of a new genus. This position was substantiated by molecular hybridization as described below. The characteristics differentiating the *Sesbania* stem-nodulating strains (*Azorhizobium*) from *Rhizobium* and *Bradyrhizobium* are summarized in Table 2. Although the *Sesbania* stem-nodulating strains cannot be distinguished from *Rhizobium* or *Bradyrhizobium* by their cell shape and size (see below), they differ from *Bradyrhizobium* and *Rhizobium* by the presence of one lateral flagellum in liquid medium (Fig. 3). Also, the morphology of their colonies on YM liquid medium or YL medium is different. *Azorhizobium caulinodans* colonies on YM liquid medium or YL medium are 1 to 2 mm in diameter and do not form slime. They also differ from *Bradyrhizobium* by their fast-growing characteristic; their generation times are 3 and 5 h in YL medium and YM liquid medium, respectively. Unlike *Rhizobium* and *Bradyrhizobium*, they do not assimilate sugars (except glucose) and possess arginine dihydrolase

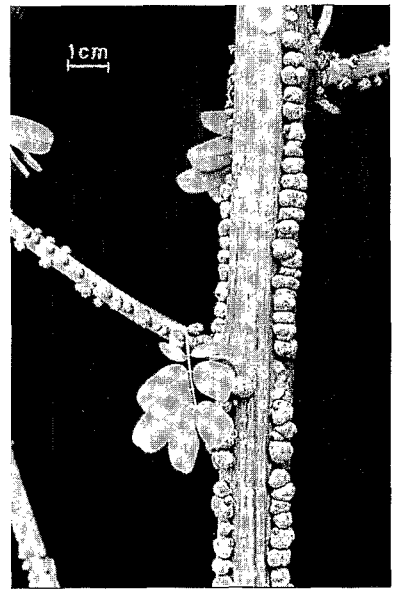

FIG. 1. Stem nodules formed by *Azorhizobium caulinodans* ORS 571^T on *S. rostrata*.

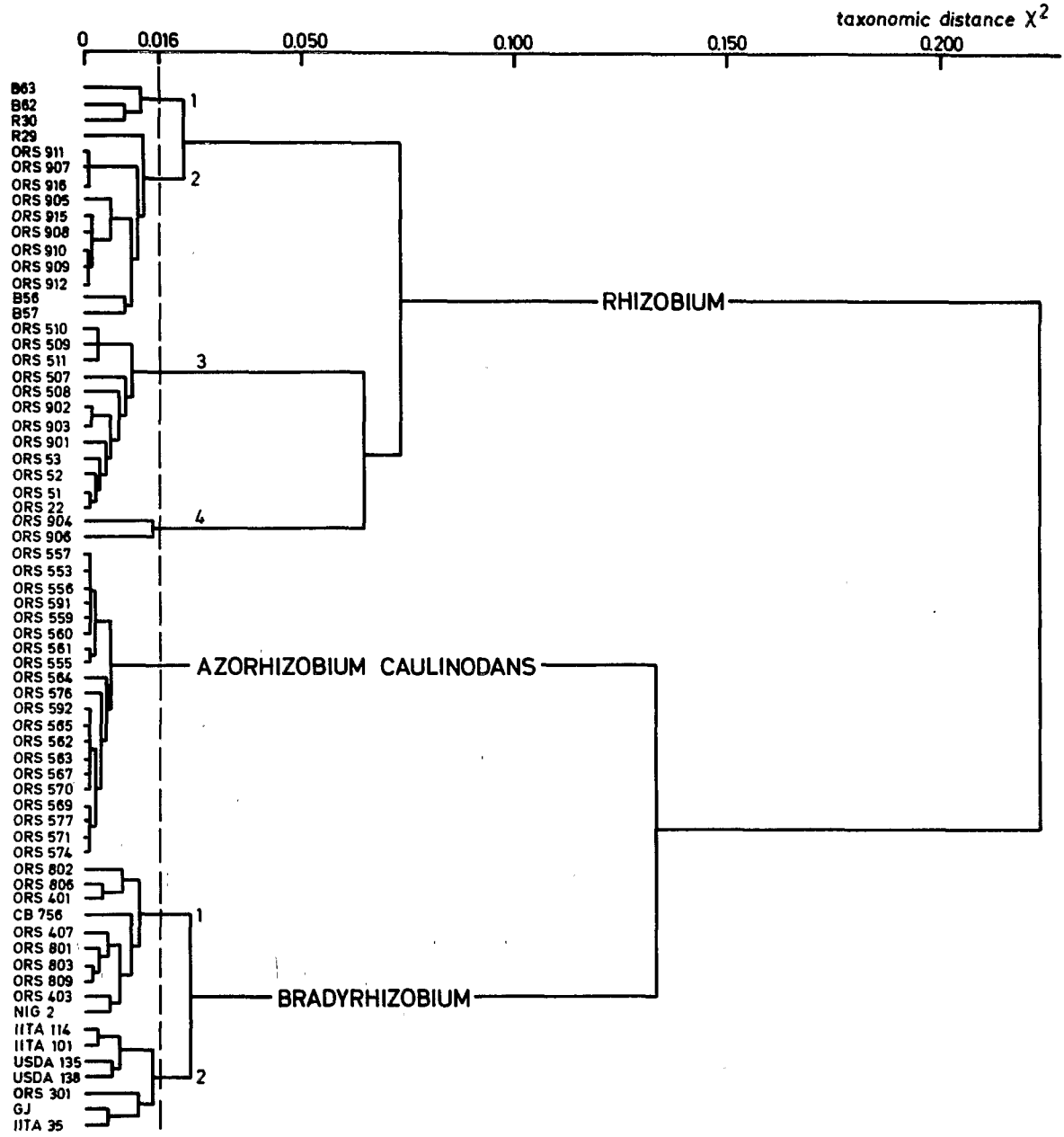

FIG. 2. Dendrogram showing the phenotypic relationships among the strains investigated, based on complete-linkage cluster analysis and calculation of chi-square values.

and lysine decarboxylase. They do assimilate alcohols such as 1,2-propanediol and 2,3-butanediol.

N_2 fixation in culture. The 20 root- and stem-nodulating strains were all able to grow on N_2 under microaerobic conditions. The nitrogenase activity was around 30 nmol of C_2H_2 produced per mg of protein per min. No growth on N_2 was detected with *Rhizobium* and *Bradyrhizobium* under similar conditions. One strain of *Bradyrhizobium japonicum*, strain GJ, and two strains of *Bradyrhizobium* sp., strains CB 756 and ORS 407, exhibited weak nitrogenase activity on soft agar cultures (5 to 10 nmol of C_2H_4 per mg of protein per h) without showing any growth. Thus, the growth on atmospheric N_2 appeared to be an important physiological discriminative character between the root- and stem-nodulating strains from *Sesbania* and strains of the genera *Rhizobium* and *Bradyrhizobium*.

Stem nodulation of *S. rostrata*. All of the strains used for the taxonomic study were tested for stem and root nodulation on *S. rostrata*. As expected, the 20 stem-nodulating strains all formed effective stem and root nodules. Among the nine root-nodulating *Rhizobium* strains, two (strains ORS 51 and ORS 507) formed pseudonodules on the stems of *S. rostrata*. Two strains, ORS 609 and ORS 611, which were not included in the numerical taxonomy analysis, were recently isolated from root nodules of *S. cannabina* and *S. grandiflora*, respectively. These two strains formed effective nodules on both roots and stems of *S. rostrata* but did not fix N_2 in culture.

DNA-rRNA hybridizations. Our phenotypic results indicated that the *Sesbania* stem-nodulating strains are quite distinct from *Rhizobium* and *Bradyrhizobium*. Preliminary DNA-rRNA hybridization studies (16) showed that *Sesbania*

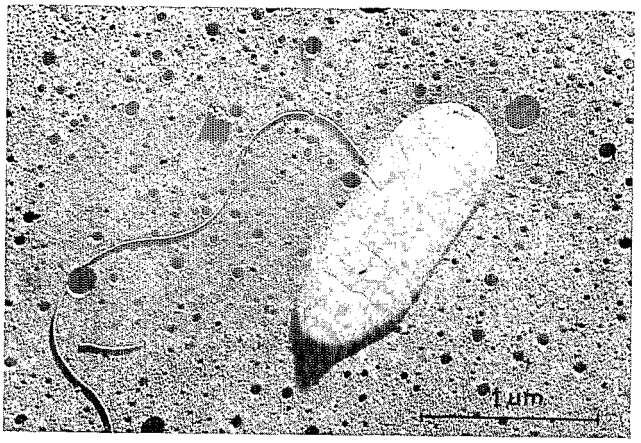

FIG. 3. Electron micrograph of negatively stained *Azorhizobium caulinodans* ORS 571^T grown in liquid medium.

stem-nodulating strain ORS 571^T is genotypically a member of the *Rhodopseudomonas palustris-Bradyrhizobium japonicum* rRNA branch in rRNA superfamily IV. With a $T_{m(e)}$ value of 73.5°C versus *Bradyrhizobium japonicum* NZP 5549^T rRNA, strain ORS 571^T appeared to be more closely related to *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris* than to all other rhizobia [$T_{m(e)}$ of 68°C versus *Bradyrhizobium japonicum* NZP 5549^T rRNA], including strains ORS 51 and ORS 22. Furthermore, unpublished data from the laboratory of J. De Ley showed that *Rhodopseudomonas acidophila*, *Rhodopseudomonas viridis*, *Methylobacterium*, *Beijerinckia*, and *Xanthobacter* have comparable $T_{m(e)}$ values versus *Bradyrhizobium japonicum* NZP 5549^T rRNA, and therefore they may be theoretically more closely related to strain ORS 571^T. In order to determine the exact taxonomic position of the free-living N_2-fixing *Sesbania* strains, we prepared [^{3}H]rRNA from strain ORS 571^T and hybridized it with DNAs from more representative *Sesbania* stem-nodulating strains and from the above-mentioned, possibly related bacteria. After sucrose gradient centrifugation the 23S [^{3}H]rRNA fraction was used for DNA-rRNA hybridizations; its specific activity was 25,400 cpm/μg. Control hybridizations were also performed with the 23S [^{14}C]rRNA fraction from *Bradyrhizobium japonicum* NZP 5549^T (16). The results are shown in Table 3, and Fig. 4 shows a $T_{m(e)}$ dendrogram. The most significant and useful parameter of a DNA-rRNA hybrid is its $T_{m(e)}$ (5, 6, 28) because this parameter is a measure of the base sequence similarity between rRNA cistrons and has decisive taxonomic significance. Our results show clearly that *Beijerinckia* and the *Sesbania* stem-nodulating strains each constitute a separate rRNA subbranch on the *Rhodopseudomonas palustris-Bradyrhizobium japonicum* rRNA branch; they split off at a mean $T_{m(e)}$ of 70.4 ± 0.6°C, indicating that their rRNA cistrons have diverged less from one another and from *Rhodopseudomonas palustris* and *Bradyrhizobium* than they have from the other branches of rRNA superfamily IV. Organisms for which the $T_{m(e)}$ differences are 10°C or more are related only beyond the genus level (5, 6, 15, 16). Four representative *Sesbania* stem-nodulating strains constitute a very narrow cluster [$T_{m(e)}$ range, 80.8 to 81.6°C] on their subbranch (Fig. 4). Among all of the possibly related organisms only the genus *Xanthobacter* appears to also be a member of this subbranch [$T_{m(e)}$ range, 77.2 to 78°C] and is consequently the closest relative of the *Sesbania* stem-nodulating strains. The latter strains cannot be included in *Xanthobacter* because a $\Delta T_{m(e)}$ of 4°C reflects an intergeneric relationship, provided sufficient phenotypic arguments are available (5, 6, 15, 16). *Xanthobacter* (30) includes rod-shaped cells which become pleomorphic on certain media; these organisms are yellow-pigmented, nitrogen-fixing, autotrophic, hydrogen-oxidizing bacteria. This genus contains two species, *Xanthobacter autotrophicus* and *Xanthobacter flavus*, of which we included three representative strains (Fig. 4). The *Sesbania* stem-nodulating strains can be differentiated from *Xanthobacter* by the absence of pleomorphy, by their motility, by their Gram reaction, by their colony morphology, and by some biochemical features (Table 4). Their G+C contents are in the same range as those of *Xanthobacter* (65 to 70 mol%).

Comparative gel electrophoresis of whole-cell proteins. We included three representative strains from the *Azorhizobium*

TABLE 3. Properties of DNA-rRNA hybrids determined by using labeled rRNAs from *Azorhizobium caulinodans* ORS 571^T and *Bradyrhizobium japonicum* NZP 5549^T

Organism used for DNA isolation	G+C content (mol%)	Hybridized with: [^{3}H]rRNA from *Azorhizobium caulinodans* ORS 571^T		Hybridized with: [^{14}C]rRNA from *Bradyrhizobium japonicum* NZP 5549^T	
		$T_{m(e)}$ (°C)	rRNA binding (%)	$T_{m(e)}$ (°C)	rRNA binding (%)
Azorhizobium caulinodans ORS 571^T	66	81.6	0.16	71.6	0.07
Azorhizobium caulinodans ORS 590	68	80.8	0.17		
Azorhizobium caulinodans ORS 591	66	80.8	0.15		
Azorhizobium caulinodans ORS 592	67	81.3	0.16	71.2	0.08
X. autotrophicus LMG 7043^T	67.3	77.3	0.10		
X. autotrophicus LMG 7044		76.4	0.09		
Xanthobacter flavus LMG 7045^T	68.1	78.0	0.15		
Beijerinckia indica NCIB 8712^T	56.9	71.0	0.10	71.6	0.09
Bradyrhizobium sp. strain NZP 2309		70.0	0.06	81.7[a]	0.04[a]
Bradyrhizobium sp. strain NZP 2314		71.0	0.06	81.5[a]	0.04[a]
Methylobacterium radiotolerans IAM 12099	64.7	68.3	0.14	71.5	0.10
Methylobacterium sp. strain LMG 6085	71.1	70.0	0.21	71.4	0.15
Rhodopseudomonas viridis DSM 135	67.4	70.2	0.13	70.1	0.10
Rhodopseudomonas acidophila GD14	65	69.2	0.16	69.0	0.14
Rhodopseudomonas acidophila ATCC 25092^T	64	69.0	0.10	69.2	0.09

[a] Data are from reference 16.

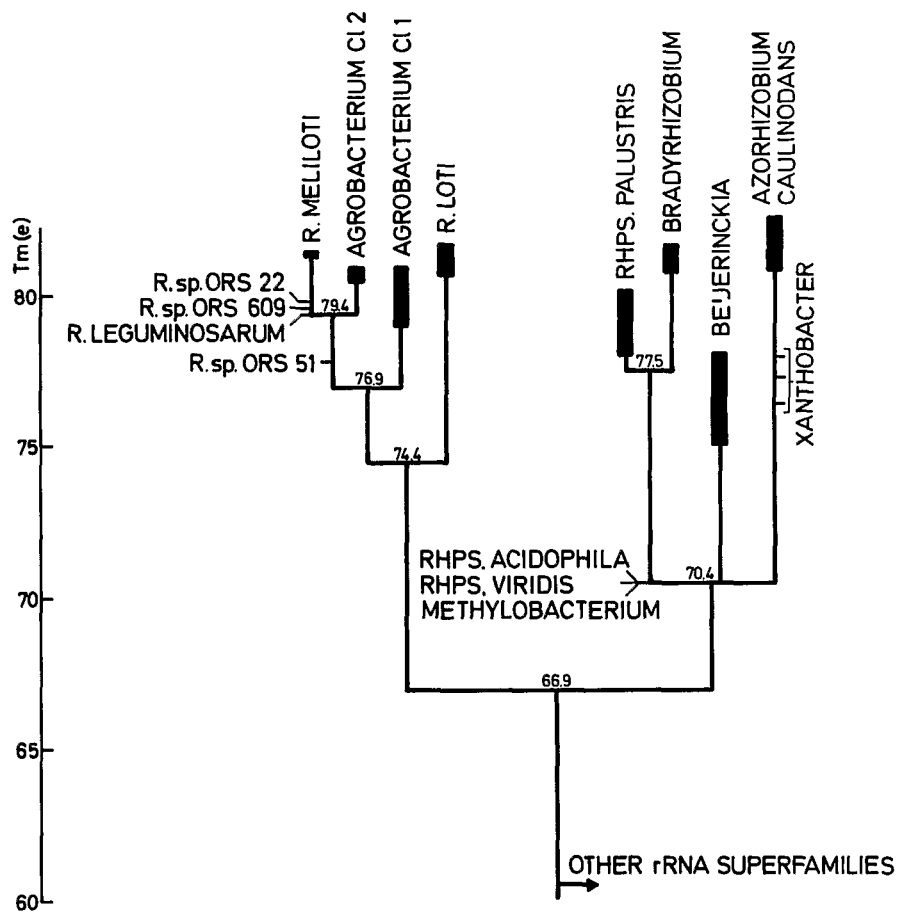

FIG. 4. Simplified rRNA cistron similarity dendrogram of part of rRNA superfamily IV, based on $T_{m(e)}$ values of DNA-rRNA hybrids. See references 16 and 25. The tops of the vertical lines represent the $T_{m(e)}$ values of the homologous duplexes of labeled reference rRNAs. The solid bars indicate the ranges observed within a given species or small group. Abbreviations: R., *Rhizobium*; Cl, cluster; RHPS., *Rhodopseudomonas*.

TABLE 4. Phenotypic features differentiating *Azorhizobium* from *Xanthobacter*[a]

Feature	*Azorhizobium*	*Xanthobacter*
Cell diam (μm)	0.5–0.6	0.4–0.8
Cell length (μm)	1.5–2.5	0.8–4
Pleomorphy	−	+
Motility	+	−
Growth at 45°C	20[b]	3
Colonies cream	20	0
Colonies yellow	0	35
Colonies filiform	0	35
Gram reaction		
Weakly positive	0	35
Negative	20	0
Growth on:		
Malonate	20	0
DL-Proline	19	1
Glucose	20	0
Fructose	0	28
Sucrose	0	24
Methanol	0	34
Ethanol	0	34

[a] Our results for 20 *Azorhizobium* strains were compared with the results of Wiegel et al. (31) and Wiegel and Schlegel (30) for 35 *Xanthobacter* strains.
[b] Numbers of strains giving positive reactions.

cluster shown in Fig. 2 (strains ORS 571^T, ORS 591, and ORS 592) and one strain (ORS 590) which was isolated later. The four stem-nodulating strains had almost identical protein gel electropherograms (Fig. 5), indicating that they constitute a very homogeneous cluster with most likely high internal DNA-DNA homologies (19). Their electropherograms are quite different from those of *Rhizobium meliloti* LMG 6130, *Bradyrhizobium japonicum* NZP 5549^T, *Xanthobacter autotrophicus* LMG 7043^T and LMG 7044, *Xanthobacter flavus* LMG 7045^T, and strain ORS 51, which nodulates only roots of *S. rostrata*. Strains ORS 609 and ORS 611, which form effective nodules on both roots and stems of *S. rostrata* but which do not fix N_2 in culture, have almost identical electropherograms, which are quite different from the electropherograms of all other strains included in this study. The DNA-rRNA hybridization results [$T_{m(e)}$ of 79.7°C versus *Rhizobium meliloti* NZP 4009 rRNA] show clearly that strains ORS 609 and ORS 611 belong in the *Rhizobium-Agrobacterium* complex. Thus, a few *Rhizobium* strains are also able to nodulate the stems of *S. rostrata*, but unlike the *Azorhizobium* strains, they do not fix N_2 in culture.

DNA-DNA hybridizations. As expected, representative strains of *Azorhizobium* have high levels of DNA binding (95% between strains ORS 571^T and ORS 591) and consequently belong in one species. With DNA from the three *Xanthobacter* strains, no measurable DNA binding (less

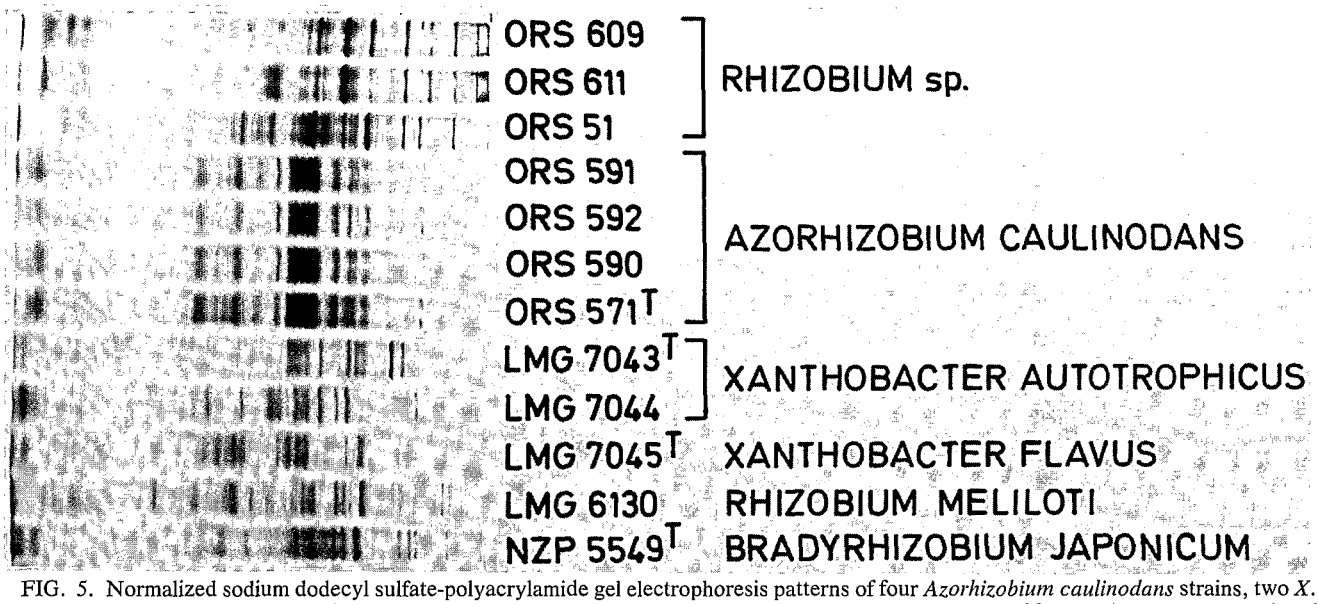

FIG. 5. Normalized sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of four *Azorhizobium caulinodans* strains, two *X. autotrophicus* strains (type strain included), the type strain of *X. flavus*, a representative *Rhizobium meliloti* strain, the type strain of *Bradyrhizobium japonicum*, and three strains of *Rhizobium* sp. isolated from root nodules of *Sesbania* sp.

than 20%) was found, as expected from their $T_{m(e)}$ values versus rRNA from *Azorhizobium caulinodans* ORS 571^T.

The reasons why we propose a new genus (*Azorhizobium*) with one species (*Azorhizobium caulinodans*) for the *Sesbania* stem-nodulating strains are summarized below. (i) The DNA-rRNA hybridization results show clearly that the *Sesbania* stem-nodulating strains constitute a separate rRNA subbranch and do not belong in *Rhizobium* or *Bradyrhizobium* [$\Delta T_{m(e)}$ of 12 and 10°C, respectively]. *Xanthobacter* strains appeared to be the closest relatives of these organisms, from which they are genotypically [$\Delta T_{m(e)}$ of 4°C] and phenotypically sufficiently different to deserve a separate generic rank.

(ii) In this new genus the *Sesbania* stem-nodulating strains constitute one species because they have almost identical protein electropherograms, show high levels of DNA binding, and constitute a phenotypically narrow cluster. A detailed description of the new genus is given below.

***Azorhizobium* gen. nov.** *Azorhizobium* (A.zo.rhi.zo'bi.um. Fr. n. *azote* nitrogen; M. L. neut. n. *Rhizobium* bacterial generic name; M. L. neut. n. *Azorhizobium* rhizobium that can use nitrogen while free living) cells are gram-negative, small rods that are 0.5 to 0.6 by 1.5 to 2.5 μm. Motile. The cells have peritrichous flagella on solid medium and one lateral flagellum in liquid medium (Fig. 3). Colonies on agar are circular and have a creamy color. Obligately aerobic. Fix atmospheric N_2 under microaerobic conditions and grow well on N_2 with vitamins present in a nitrogen-free medium. Oxidase and catalase are present; urease is absent. Among the sugars, only glucose is oxidized. Organic acids such as lactate or succinate are the favorite carbon substrates for both NH_4^+- and N_2-dependent growth. Malonate is also used. Starch is not hydrolyzed. Can grow on DL-proline. No strain dinitrifies. Strains nodulate effectively the roots and stems of *S. rostrata*. The type species is *Azorhizobium caulinodans*.

***Azorhizobium caulinodans* sp. nov.** *Azorhizobium caulinodans* (cau.li'no.dans. M. L. n. *caulis* stem; M. L. v. *nodare* to nodulate; M. L. part. adj. *caulinodans* stem nodulating) cells are as described above for the genus. They effectively nodulate roots and stems of *S. rostrata*, possess arginine dihydrolase and lysine decarboxylase, and grow in the presence of 8% KNO_3. They grow on azelate, maleate, adipate, pimelate, suberate, gluconate, mucate, crotonate, nicotinate, 2-ketogluconate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate, laurate, 2-ketoglutarate, fumarate, glutarate, sebacate, DL-malate, citrate, pyruvate, aconitate, citraconitate, D-glucuronate, α-D-galacturonate, *m*-hydroxybenzoate, L-aspartate, quinate, L-α-alanine, L-lysine, L-asparagine, betaine, and sarcosine. Do not oxidize mannitol. Assimilate 1,2-propanediol and 2,3-butanediol. Growth occurs from 12 to 43°C. Among the vitamins, nicotinic acid is required for N_2 fixation under microaerobic conditions. Equally good growth is observed between pHs 5.5 and 7.8. Other physiological characteristics are as described above for the genus. The G+C content of the DNA is 66.5 mol%. Strain ORS 571 (= LMG 6465) is the type strain. The G+C content of the type strain is 66 mol%.

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LITERATURE CITED

1. **Claus, D., and C. Schaab-Engels (ed.).** 1977. Catalogue of strains. Deutsche Sammlung von Mikroorganismen, 2nd ed. Gesellschaft für Strahlen- und Umweltforschung mbH, Munich.
2. **De Ley, J.** 1970. Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. J. Bacteriol. **101**:738–754.
3. **De Ley, J., and R. Tytgat.** 1970. Evaluation of membrane filter methods for DNA:DNA hybridizations. Antonie van Leeuwen-

hoek J. Microbiol. Serol. **36**:193–207.
4. **De Ley, J., and J. Van Muylem.** 1963. Some applications of deoxyribonucleic acid base composition in bacterial taxonomy. Antonie van Leeuwenhoek J. Microbiol. Serol. **29**:344–358.
5. **De Smedt, J., and J. De Ley.** 1977. Intra- and intergeneric similarities of *Agrobacterium* ribosomal ribonucleic acid cistrons. Int. J. Syst. Bacteriol. **27**:222–240.
6. **De Vos, P., M. Goor, M. Gillis, and J. De Ley.** 1985. Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. Int. J. Syst. Bacteriol. **35**:169–184.
7. **Doetsch, R. N.** 1981. Determinative methods of light microscopy, p. 21–33. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
8. **Dreyfus, B., D. Alazard, and Y. R. Dommergues.** 1984. Stem-nodulating rhizobia, p. 161–169. *In* M. C. Klug and C. E. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.
9. **Dreyfus, B., and Y. R. Dommergues.** 1981. Nitrogen-fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata*. FEMS Microbiol. Lett. **10**:313–317.
10. **Dreyfus, B. L., C. Elmerich, and Y. R. Dommergues.** 1983. Free-living *Rhizobium* strains able to grow on N_2 as the sole nitrogen source. Appl. Environ. Microbiol. **45**:711–713.
11. **Garcia, J. L., S. Roussos, M. Bensoussan, A. Bianchi, and M. Mandel.** 1982. Taxonomie numérique de *Bacillus* thermophiles isolés de sols de rhizière de l'Ouest. Ann. Inst. Pasteur Paris **133A**:471–488.
12. **Garcia, J. L., S. Roussos, D. Gauthier, G. Rinaudo, and M. Mandel.** 1983. Etude taxonomique de bactéries azotofixatrices libres isolées de l'endorhizosphère du riz. Ann. Inst. Pasteur Paris **134B**:329–346.
13. **Gebhardt, C., G. L. Turner, A. H. Gibson, B. L. Dreyfus, and F. J. Bergersen.** 1984. Nitrogen-fixing growth in continuous culture of a strain of *Rhizobium* sp. isolated from stem-nodules on *Sesbania rostrata*. J. Gen. Microbiol. **130**:843–846.
14. **Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.).** 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
15. **Gillis, M., and J. De Ley.** 1980. Intra- and intergeneric similarities of the ribosomal ribonucleic acid cistrons of *Acetobacter* and *Gluconobacter*. Int. J. Syst. Bacteriol. **30**:7–27.
16. **Jarvis, B. D. W., M. Gillis, and J. De Ley.** 1986. Intra- and intergeneric similarities between ribosomal ribonucleic acid cistrons of *Rhizobium* and *Bradyrhizobium* species and some related bacteria. Int. J. Syst. Bacteriol. **36**:129–138.
17. **Jordan, D. C.** 1984. Family III. *Rhizobiaceae* Conn 1938, p. 234–244. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The William & Wilkins Co., Baltimore.
18. **Juthner, R. R., R. M. Lafferty, and H. J. Knackmuss.** 1975. A simple method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. **1**:233–237.
19. **Kersters, K.** 1985. Numerical methods in the classification of bacteria by protein electrophoresis, p. 337–368. *In* M. Goodfellow, D. Jones, and F. G. Priest (ed.), Computer-assisted bacterial systematics. Academic Press, Inc. (London), Ltd., London.
20. **Kiredjian, M., B. Holmes, K. Kersters, I. Guilvout, and J. De Ley.** 1986. *Alcaligenes piechaudii*, a new species from human clinical specimens and the environment. Int. J. Syst. Bacteriol. **36**:282–287.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Lovelace, T. E., and R. R. Colwell.** 1968. A multipoint inoculator for petri dishes. Appl. Microbiol. **18**:944–945.
23. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
24. **Nishimura, Y., S. Yamanaka, and H. I. Iizuka.** 1983. Ubiquinone, fatty acid, polar lipid and DNA base composition in *Pseudomonas radiora*. J. Gen. Appl. Microbiol. **29**:421–427.
25. **Reinhold, B., T. Hurek, I. Fendrik, B. Pot, M. Gillis, K. Kersters, S. Thielemans, and J. De Ley.** 1987. *Azospirillum halopraeferens* sp. nov., a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). Int. J. Syst. Bacteriol. **37**:43–51.
26. **Rhodes, M. E.** 1958. The cytology of *Pseudomonas* spp. as revealed by a silver-plating staining method. J. Gen. Microbiol. **18**:639–648.
27. **Richards, G. M.** 1974. Modification of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal. Biochem. **57**:369–376.
28. **Van Landschoot, A., and J. De Ley.** 1983. Intra- and intergeneric similarities of the rRNA cistrons of *Alteromonas*, *Marinomonas* (gen. nov.) and some other gram-negative bacteria. J. Gen. Microbiol. **129**:3057–3074.
29. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria, p. 73–97. International Biological Programme Handbook. Blackwell Scientific Publications, Ltd., Oxford.
30. **Wiegel, J. K. W., and H. G. Schlegel.** 1984. Genus *Xanthobacter* Wiegel, Wilke, Baumgarten, Opitz and Schlegel, p. 325–333. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
31. **Wiegel, J., D. Wilke, J. Baumgarten, R. Opitz, and H. G. Schlegel.** 1978. Transfer of the nitrogen-fixing hydrogen bacterium *Corynebacterium autotrophicum* Baumgarten et al. to *Xanthobacter* gen. nov. Int. J. Syst. Bacteriol. **28**:573–581.