INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, Jan. 1991, p. 114–120 Vol. 41, No. 1
0020-7713/91/010114-07$02.00/0

DNA Homologies among Members of the Genus *Azorhizobium* and Other Stem- and Root-Nodulating Bacteria Isolated from the Tropical Legume *Sesbania rostrata*

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The diversity among 191 bacterial strains isolated from stem and root nodules (151 and 40 strains, respectively) of *Sesbania rostrata* grown in different geographical areas in Senegal and in The Philippines was studied by using DNA-DNA hybridization techniques (S1 nuclease method), by determining DNA base compositions, by performing legume nodulation tests, and by determining nitrogenase activity. The following conclusions were drawn. (i) All of the strains produced stem and root nodules on *S. rostrata*. (ii) Most of the organisms (184 strains) belonged to the genus *Azorhizobium*; their guanine-plus-cytosine contents ranged from 66 to 68 mol%, they fixed N_2 under free-living conditions, and they produced effective nodules on the stems and roots of *S. rostrata*. (iii) The seven other strains probably belonged to the genus *Rhizobium*, since guanine-plus-cytosine contents ranged from 59 to 63 mol% and they did not fix N_2 under free-living conditions; three strains produced effective root nodules, but their stem nodules exhibited very low activity or were ineffective, and the four remaining strains produced ineffective nodules on both stems and roots. (iv) The genetic diversity among the 184 *Azorhizobium* strains allowed us to divide them into two genomic species; genomic species 1 constituted the major group (175 strains) and corresponded to *Azorhizobium caulinodans* since all of the strains were more than 79% related to type strain ORS 571, and genomic species 2 contained nine strains that were only 44 to 53% related to type strain ORS 571 (difference between the denaturation temperatures of homologous and heterologous hybrids, more than 6°C) and more than 76% related to reference strains SD02 and SG28 (difference between the denaturation temperatures of homologous and heterologous hybrids, less than 3°C). The species that were distinct from *A. caulinodans* cannot be named until they can be differentiated by phenotypic tests.

The symbiosis between legumes and bacteria that are able to induce formation of nitrogen-fixing nodules has attracted attention because of its importance in agriculture and its suitability as a research system involving procaryotic and eucaryotic partners.

In *Bergey's Manual of Determinative Bacteriology*, 8th ed., all of the bacteria which induce nitrogen-fixing nodules on leguminous plants were included in one genus, the genus *Rhizobium* (17). These organisms were later divided into two genera, *Rhizobium* (fast growers) and *Bradyrhizobium* (slow growers) (15, 16). Both of these genera are considered to be members of the family *Rhizobiaceae*, but they are quite distinct in their genetic and physiological characteristics. The genus *Rhizobium* includes three fast-growing species, *Rhizobium leguminosarum*, *Rhizobium meliloti*, and *Rhizobium loti*, which form nodules on roots of leguminous plants that grow predominantly in temperate zones; it no longer includes the species *Rhizobium fredii* (27), which has been placed in the new genus *Sinorhizobium*. The genus *Sinorhizobium* contains two species, *Sinorhizobium fredii* and *Sinorhizobium xinjiangensis* (3). The genus *Bradyrhizobium* comprises one well-defined species, *Bradyrhizobium japonicum*, and includes all of the bacteria that were referred to previously as slow-growing rhizobia. The bacteria belonging to the genus *Bradyrhizobium* form nodules on roots of tropical leguminous plants and some temperate leguminous plants.

A study of bacteria that are able to produce N_2-fixing nodules both on the roots and on the stems of the tropical legume *Sesbania rostrata* led to the proposal of the new genus *Azorhizobium*, which is quite distinct from the genera *Rhizobium* and *Bradyrhizobium*, and its one species, *Azorhizobium caulinodans* (type strain ORS 571) (7). Previous DNA-rRNA hybridization experiments (13) have shown that stem- and root-nodulating strain ORS 571^T (T = type strain) is genotypically a member of the *Rhodopseudomonas palustris-B. japonicum* rRNA branch in rRNA superfamily IV sensu De Ley (5). The *Sesbania* stem-nodulating strains constitute a separate rRNA subbranch; the closest relative of these organisms is the genus *Xanthobacter*. The *Azorhizobium* strains have the unusual feature that they grow rapidly in the free-living state at the expense of N_2 as the sole source of nitrogen when they are incubated under microaerobic conditions (6, 8). In addition to *Azorhizobium* strains, *Sesbania rostrata* can also be associated with root-nodulating strains which do not fix nitrogen in culture and are genuine rhizobia (7).

The purposes of our work were to examine the genetic diversity in the genus *Azorhizobium* by using DNA-DNA hybridization techniques and by determining DNA base compositions and to assess the ability of the strains to fix nitrogen in culture or symbiotically with the host plant. We analyzed a large collection of strains that were isolated from stem and root nodules of *Sesbania rostrata* plants located in various geographical areas in Senegal and The Philippines.

The results of this work led to the identification of two

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TABLE 1. Origin of strains tested

Strain(s)[a]	Location	Isolated from:
Strains from North Senegal		
A. caulinodans ORS 571^T (= LMG 6465^T)		Stem nodules
SV01 to SV30	Savoigne	Stem nodules
SV31 to SV34	Savoigne	Root nodules
BD01 to BD03	Boundoum	Stem nodules
BD04, BD05	Boundoum	Root nodules
RT01 to RT13	Richard Toll	Stem nodules
RT14 to RT18	Richard Toll	Root nodules
FY01 to FY24	Fanaye	Stem nodules
FY25 to FY30	Fanaye	Root nodules
PR01	Podor	Stem nodules
Strains from Central Senegal		
SG01 to SG25	Senghor	Stem nodules
SG26 to SG32	Senghor	Root nodules
DP01 to DP19	Diouroup	Stem nodules
DP20 to DP23	Diouroup	Root nodules
KL01 to KL15	Kaolack	Stem nodules
KL16 to KL26	Kaolack	Root nodules
SD01 to SD04	Sandiara	Stem nodules
SK01 to SK05	Sebikotane	Stem nodules
Strains from the Philippines		
IRG10, IRG13, IRG19, IRG22, IRG32, IRG40, IRG42, IRG44, IRG45, IRG46		Stem nodules
IRG23		Root nodules
TAL 674[b]		Stem nodules

[a] All of the strains from Senegal except strain ORS 571^T were our isolates; strain ORS 571^T was obtained from the Collection of Bacteria of the Laboratorium voor Microbiologie, Ghent, Belgium. Strain TAL 674 and the other strains from The Philippines were received from J. K. Ladha, International Rice Research Institute, Manila, The Philippines.

[b] Strain from the NifTAL Culture Collection.

genomic species in the genus *Azorhizobium* and seven unclassified strains, which are probably genuine rhizobia.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains included in this investigation are listed in Table 1. All of the strains were grown on yeast extract-lactate medium containing (per liter of distilled water) 0.5 g of K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 0.005 g of $FeCl_3$, 1.0 g of yeast extract (Difco), 10 g of sodium lactate, and 15 g of agar (Difco); the pH of this medium was 6.8.

To prepare semisolid YLO medium, which was used for nitrogenase assays, ammonium sulfate was omitted from yeast extract-lactate medium and the concentrations of yeast extract and agar were decreased to 0.05 and 3 g/liter, respectively.

DNA extraction and purification. DNA was extracted and purified by using the procedure of Brenner et al. (2).

Determination of base composition. The average guanine-plus-cytosine (G+C) contents of the DNAs of the strains investigated were determined by a high-performance liquid chromatography method previously used for staphylococci and micrococci (23) and for strains of the genus *Frankia* (9), using the method of Gehrke et al. (10). The Merck Hitachi chromatography system used for quantification of nucleotides included a pump (catalog no. L-6200), a UV detector (catalog no. L-4000) linked to an integrator (catalog no. D-2500), and a 7-μm type RP18 chromatography column (catalog no. 15539); the mobile phase was 0.6 M $NH_4H_2PO_4$, the pH was 4.25, the flow rate was 1.0 ml/min, and absorption was measured at 260 nm.

DNA-DNA hybridization and thermal stability of duplexes. DNA-DNA hybridization tests were carried out at 70°C (the optimal temperature for DNA reassociation) with tritium-labeled DNAs from strains ORS 571^T, SD02, SG28, RT12, RT14, and TAL 674 by using the S1 nuclease-trichloroacetic acid procedure of Grimont et al. (12). Native DNAs were labeled by using the nick translation reaction (24) in the presence of free tritium-labeled nucleotides (Amersham International, Amersham, England).

The thermal stability of reassociated DNAs was estimated by determining the denaturation temperature (T_m) (the temperature at which 50% of the double-stranded DNA was denaturated and lysed by S1 nuclease). The T_m was determined by using the method of Crosa et al. (4), slightly modified (9). The divergence between DNAs was estimated by determining the ΔT_m value (the difference between the T_m values of homologous and heterologous hybrids).

Nitrogenase assays. The abilities of the strains to fix nitrogen in the free-living state or symbiotically with *Sesbania rostrata* were estimated by using the acetylene reduction assay; ethylene production was detected with a flame ionization gas chromatograph (type 30.F.PT Girdel chromatograph). Tests with pure cultures were carried out in 10-ml rubber-capped tubes containing 3 ml of semisolid YLO medium. Cultures were incubated in the presence of 2% C_2H_2 in the gas phase. Gas samples were analyzed for C_2H_4 production after 2 days.

Infection tests were performed by inoculating stems and roots of 4-week-old plants with strains grown on yeast extract-lactate liquid medium for 48 h. The effectiveness of the symbiosis was estimated 3 weeks after inoculation by incubating nodulated stems or root systems in a 10% C_2H_2–90% air mixture. Gas samples were analyzed for C_2H_4 production after 30 min of incubation.

RESULTS

The G+C contents of *Azorhizobium* strains range from 66 to 68 mol%. In addition, the ability to grow at the expense of atmospheric nitrogen as the sole source of nitrogen is an important characteristic which allows discrimination between *Azorhizobium* strains and strains of the genera *Rhizobium* and *Bradyrhizobium*; some *Bradyrhizobium* strains have been shown to be able to reduce acetylene under microaerobic conditions, but these organisms require a source of combined nitrogen to support growth (18, 19, 21, 22). These observations led us to classify the strains which we studied as either typical *Azorhizobium* strains or other strains in order to facilitate interpretation of the results which we obtained (Tables 2 through 4).

DNA analyses. The G+C contents and percentages of relative DNA homology at 70°C with reference DNAs from strains ORS 571^T, SD02, SG28, RT12, RT14, and TAL 674 are shown in Table 2 for 66 strains. Results for the other 125 strains, for which only reassociation data were obtained, are shown in Table 3.

Of the 191 strains tested, 175 strains fell into a large DNA relatedness group, genomic species 1. These strains were at least 79% related to *A. caulinodans* type strain ORS 571 and exhibited low levels of divergence (ΔT_m, less than 2°C), and their G+C contents ranged from 66 to 68 mol%.

Genomic species 2 contained nine strains which had G+C contents of 66 to 67 mol%. These organisms were more than 76% related to strain SD02 (ΔT_m, less than 3°C) and more

TABLE 2. Levels of DNA hybridization among strains

Source of unlabeled DNA	G+C content (mol %)	% Reassociation at 70°C with labeled DNA from: Strain ORS 571^T	Strain SD02	Strain SG28	Strain RT12	Strain RT14	Strain TAL 674
Typical *Azorhizobium* strains							
Genomic species 1							
ORS 571^T	67	100[a]	47	49	2	5	
SV01	66	91					
SV06	66	98					
SV07	67	103		53			
SV08	66	96					
SV20	66	96					
SV25	68	84	53	55			
SV26		96			3		
SV31	68	87					
SV33		87 (1.3)[b]					
SV34		95	58				
BD01	66	98					
BD05	67	92					
RT01	66	88					
RT06	67	94					
RT10		104	51	46			
RT15	66	84					
RT18	67	92	51				
FY01	66	93					
FY10	68	81					
FY18	67	96					
FY29	67	89	48	50			
PR01	66	97	55	44			
SG01	66	97					
SG07	67	82					
SG10	67	94					
SG24	67	91					
SG26	66	85					
SG27	67	101	49	54			
SG31		106	53				
DP04	67	92					
DP13	67	92					
DP18	66	98	57	53			
DP19	67	105					
DP20		85	57				
DP22	67	98	55				
DP23	67	81					
KL03	66	94					
KL06	67	82 (1.5)	51 (17.8)				
KL11	68	99	53		2	7	4
KL14	66	90					
KL16	66	101					
KL17	67	92	59	58			
SD01	67	104					
SD03	67	83 (1.7)	55 (15.2)		2		
SK02	66	99					
IRG10	67	92					
IRG40		89	50				
IRG44	68	102					
IRG23	67	100					
Genomic species 2							
SD02	67	53 (8.8)	100	87			
SD04	67	44 (7.9)	86		5		
SG28	66	47 (7.2)	92	100			
SG05	67	44 (9.1)	79 (2.2)	90	5		
SG06	66	46 (9.7)	94		7		
SG09	66	51 (6.7)	76 (1.6)	92			
SG19	66	51 (6.6)	83 (3.0)				
SG22	67	48 (7.0)	89	86			
SG25	67	50 (6.4)	90				
Other strains							
Genomic species 3							
RT12	63	0	2	1	100		
Genomic species 4							
RT14	60	4	0	1	3	100	7

Continued on following page

TABLE 2—*Continued*

Source of unlabeled DNA	G+C content (mol %)	% Reassociation at 70°C with labeled DNA from:					
		Strain ORS 571[T]	Strain SD02	Strain SG28	Strain RT12	Strain RT14	Strain TAL 674
Genomic species 5							
TAL 674	59	4	3	2	11		100
Unclassified							
RT09	61	4	0	1	5	10	7
RT11	61	2	−1	2	5	10	10
DP21	61	0	6	1	6	8	10
KL13	61	−2	4	6	−2	9	15

[a] Level of relatedness at 70°C.
[b] The numbers in parentheses are ΔT_m values (in degrees Celsius).

than 86% related to strain SG28 (Table 2). These strains were only 44 to 53% related to strain ORS 571[T] (ΔT_m, 6.4 to 9.7°C).

The remaining seven strains (strains RT12, RT14, TAL 674, RT09, RT11, DP21, and KL13) had lower G+C contents (59 to 63 mol%) and exhibited very low levels of DNA binding with strain ORS 571[T] (less than 4%) or strains SD02 and SG28 (less than 6%) (Table 2). Strains RT12, RT14, and TAL 674 constituted three distinct groups (genomic species 3, 4, and 5). Strains RT09, RT11, DP21, and KL13 exhibited very low levels of DNA binding with strains RT12, RT14, and TAL 674 (0 to 15%) and remained unclassified.

Plant infection tests and nitrogenase activity. All of the strains were able to nodulate both roots and stems of *Sesbania rostrata* (Table 4). However, on the basis of the results of the acetylene reduction tests, we observed differences in the ability of the strains to fix nitrogen under free-living conditions or symbiotically.

All of the strains belonging to genomic species 1 (only the results obtained with strain ORS 571[T] are reported here) and genomic species 2 expressed nitrogenase activity both in vitro (free-living conditions) and under symbiotic conditions (in stem and root nodules).

The seven other strains, which were characterized by lower G+C contents, did not reduce acetylene under free-living conditions. In symbiosis with *Sesbania rostrata*, strains RT12, RT14, and TAL 674 formed effective root nodules, but their stem nodules exhibited very low activity (strains RT12 and RT14) or were ineffective (strain TAL 674), while strains RT09, RT11, DP21, and KL13 formed ineffective nodules on both roots and stems.

DISCUSSION

A large proportion (92%) of the strains which we analyzed were members of genomic species 1, which corresponds to *A. caulinodans* since it contains type strain ORS 571 (Tables 2 and 3). This group was not limited to one geographic area; it contained 90% of the strains that were isolated from 10 distinct stations in North or Central Senegal (Table 1) and all of the strains from The Philippines. The type of nodules (stem or root nodules) from which strains were isolated seems not to have had any selective effect; 90 and 92% of the strains that were isolated from stem and root nodules, respectively, belonged to genomic species 1. The ability to grow at the expense of atmospheric N_2 as the sole source of nitrogen allowed us to discriminate between *Azorhizobium* strains and strains belonging to the genera *Rhizobium* and *Bradyrhizobium*. As expected, the genomic species 1 strains expressed nitrogenase activity both in vitro and in symbiosis with the host plant.

According to Grimont (11), strains that exhibit less than 60% reassociation and more than 7°C divergence do not belong to the same genomic species. Thus, the results obtained with the nine strains belonging to genomic species 2 showed that these organisms are sufficiently different from genomic species 1 that they constitute a new species. However, this genomic species cannot be named until it can be differentiated by some phenotypic property, as recommended by the International Committee on Systematic Bacteriology (28). The genomic species 2 strains had the same N_2-fixing properties as the genomic species 1 strains (i.e., the ability to fix nitrogen in culture and the ability to produce effective nodules on the stems and roots of *Sesbania rostrata*). They were all isolated from two stations in Central Senegal (Sandiara and Senghor). It must be pointed out that all of the strains from The Philippines belonged to the species *A. caulinodans*. Distant geographical origins did not seem to be correlated with genetic diversity. Such an observation is consistent with the findings of Dreyfus et al. (7), who reported that strain ORS 591 from Madagascar exhibited a high level of DNA binding (95%) with strain ORS 571[T]. However, all of the seeds of *Sesbania rostrata* introduced into The Philippines originated from Senegal (26); thus, azorhizobia may have been introduced at the same time. Such natural seed contaminants have been documented previously (20).

The remaining strains (strains RT12, RT14, TAL 674, RT09, RT11, DP21, and KL13) produced profuse stem nodules and root nodules on *Sesbania rostrata* (Table 4), but were quite different from typical *Azorhizobium* strains; they had lower G+C contents (59 to 63 mol%) and did not fix N_2 under free-living conditions. DNA relatedness data showed that, unlike members of the genus *Azorhizobium*, the members of this group are highly heterogeneous, since among the seven strains at least four genomic species could be differentiated. These strains could be compared with strains ORS 51, ORS 609, and ORS 611, which were isolated from root nodules of *Sesbania rostrata*, *Sesbania cannabina*, and *Sesbania grandiflora*, respectively and were reported to form stem and root nodules on *Sesbania rostrata*, but, unlike the *Azorhizobium* strains, could not fix N_2 in culture; DNA-rRNA hybridization results showed that strains ORS 51 and ORS 609 are members of the *Rhizobium-Agrobacterium* rRNA branch (7).

Our results showed that azorhizobia represented a very large proportion of our collection of stem- and root-nodulating strains. It has been established that some legumes can be

TABLE 3. Levels of DNA hybridization between strain ORS 571^T and typical *Azorhizobium* strains belonging to genomic species 1

Source of unlabeled DNA	% Reassociation at 70°C with labeled DNA from strain ORS 571^T	Source of unlabeled DNA	% Reassociation at 70°C with labeled DNA from strain ORS 571^T
SV02	97	SG03	96
SV03	85	SG04	82
SV04	94	SG08	98
SV05	85	SG11	90
SV09	95	SG12	79
SV10	82	SG13	97
SV11	84	SG14	99
SV12	101	SG15	81
SV13	88	SG16	100
SV14	82	SG17	85
SV15	81	SG18	88
SV16	89	SG20	87
SV17	97	SG21	97
SV18	90	SG23	83
SV19	100	SG29	87
SV21	95	SG30	97
SV22	88	SG32	93
SV23	91	DP01	80
SV24	86	DP02	87
SV27	98	DP03	88
SV28	97	DP05	99
SV29	103	DP06	84
SV30	83	DP07	97
SV32	90	DP08	94
BD02	99	DP09	83
BD03	96	DP10	85
BD04	84	DP11	95
RT02	93	DP12	98
RT03	90	DP14	84
RT04	87	DP15	93
RT05	105	DP16	95
RT07	93	DP17	97
RT08	90	KL01	91
RT13	87	KL02	99
RT16	80	KL04	86
RT17	99	KL05	80
FY02	88	KL07	87
FY03	88	KL08	96
FY04	88	KL09	102
FY05	86	KL10	90
FY06	93	KL12	97
FY07	101	KL15	97
FY08	95	KL18	83
FY09	98	KL19	86
FY11	88	KL20	92
FY12	88	KL21	87
FY13	90	KL22	94
FY14	93	KL23	84
FY15	100	KL24	96
FY16	94	KL25	87
FY17	103	KL26	92
FY19	94	SK01	82
FY20	98	SK03	107
FY21	91	SK04	88
FY22	100	SK05	83
FY23	89	IRG13	100
FY24	92	IRG19	103
FY25	85	IRG22	93
FY26	87	IRG32	96
FY27	89	IRG42	96
FY28	90	IRG45	99
FY30	89	IRG46	98
SG02	98		

TABLE 4. Nitrogenase activities of strains under free-living conditions and in symbiosis with *Sesbania rostrata*

Strain	Acetylene reduction test performed in culture[a]	Symbiotic conditions		
		Stem and root nodulation[b]	Acetylene reduction activity (μmol of C_2H_4/plant/h)	
			Stems	Roots
Typical *Azorhizobium* strains				
Genomic species 1[c]				
ORS 571[T]	+	+	28 (218)[d]	7
Genomic species 2				
SD02	+	+	21 (197)	15
SD04	+	+	27 (136)	10
SG05	+	+	47 (147)	15
SG06	+	+	15 (181)	4
SG09	+	+	24 (220)	8
SG19	+	+	11 (129)	3
SG22	+	+	14 (134)	3
SG25	+	+	16 (188)	29
SG28	+	+	29 (123)	6
Other strains				
Genomic species 3				
RT12	−	+	1.4 (148)	22
Genomic species 4				
RT14	−	+	0.4 (12)	16
Genomic species 5				
TAL 674	−	+	0.0 (150)	4
Unclassified				
RT09	−	+	0.0 (127)	0.0
RT11	−	+	0.0 (173)	0.0
DP21	−	+	0.0 (120)	0.0
KL13	−	+	0.0 (144)	0.0

[a] +, Positive (acetylene reduction); −, negative (no acetylene reduction).
[b] All strains nodulated both stems and roots of *Sesbania rostrata*.
[c] All strains belonging to genomic species 1 reduced acetylene in culture and formed effective stem and root nodules on *Sesbania rostrata*.
[d] The values in parentheses are the numbers of nodules on the stems.

nodulated by organisms belonging to different genera; *Bradyrhizobium* strains produce nodules on roots of certain species belonging to the genera *Lotus*, *Vigna*, *Lupinus*, *Ornithopus*, *Cicer*, *Sesbania*, *Leucaena*, *Mimosa*, *Lablab*, and *Acacia*, together with the fast-growing organism *Rhizobium loti* (14). Another significant example is the nodulation of soybeans by *B. japonicum* and the fast-growing rhizobia included in the new genus *Sinorhizobium* (3, 27). Recent studies (1) have shown that *Azorhizobium* strains are present as epiphytic bacteria on their host plants (10^5 to 10^7 bacteria per g [dry weight] of leaves and flowers). This ecological adaptation to epiphytic growth and survival may explain the predominance of *Azorhizobium* strains among a bacterial population (including rhizobia) that is able to produce both stem and root nodules.

Because of its exceptional N_2-fixing potential, the *Azorhizobium-Sesbania rostrata* symbiosis is particularly important in tropical agriculture (25). However, significant differences can be observed among *Azorhizobium* strains when their abilities to nodulate and fix nitrogen symbiotically with *Sesbania rostrata* are compared. Within genomic species 2, for example, the stem nodule acetylene reduction activities of strains SG19 and SG05 were 11 and 47 μmol of C_2H_4 per h per plant, respectively (Table 4). Similar results were obtained in The Philippines (20). Such differences in symbiotic efficiency show that further investigations will be necessary to obtain a better understanding of the diversity among members of the genus *Azorhizobium*.

ACKNOWLEDGMENTS

We thank L. Gay for scintillation counting, J. Freney for professional advice, J. K. Ladha for giving us strains from The Philippines, and P. A. D. Grimont for critical reading of the manuscript.

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