Intra- and Intergeneric Similarities of Ribosomal Ribonucleic Acid Cistrons of Free-Living, Nitrogen-Fixing Bacteria

J. DE SMEDT, M. BAUWENS, R. TYTGAT, AND J. DE LEY

Laboratorium voor Microbiologie en Microbiële Genetica, Faculteit der Wetenschappen, Rijksuniversiteit, B-9000 Gent, Belgium

¹⁴C-labeled ribosomal ribonucleic acid (rRNA) was prepared from *Azotobacter chroococcum* NCIB 8002, *Azotobacter paspali* 8A, *Azomonas agilis* NCIB 8636, *Azomonas insignis* WR 30, *Beijerinckia indica* NCIB 8712, and *Azospirillum brasilense* ATCC 29145. These rRNA's were hybridized under stringent conditions with filter-fixed deoxyribonucleic acid from a great variety of gram-negative bacteria. Each hybrid was described by: (i) the temperature at which 50% of the hybrid was denatured, and (ii) the percent rRNA binding (amount in micrograms of rRNA duplexed to 100 μ g of deoxyribonucleic acid). These data were used to construct rRNA similarity maps. The following conclusions could be drawn concerning rRNA cistron similarities. (i) Bacterial genera with free-living, aerobic, nitrogen-fixing members are very diverse and belong to different rRNA superfamilies. The present family *Azotobacteriaceae* is not a biological unit, and its status as a family is highly questionable. (ii) *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Azotobacter beijerinckii*, *Azotobacter paspali*, *Azotobacter miscel-lum*, *Azotobacter armeniae*, and *Azotobacter nigricans* belong in the genus *Azotobacter*. Any synonymy of these names remains to be determined. *Azomonas agilis*, *Azomonas insignis*, and *Azomonas macrocytogenes* constitute independent branches, which are about equidistant from *Azotobacter* and section I of *Pseudomonas* as presented in *Bergey's Manual of Determinative Bacteriology*, 8th ed. *Xanthomonas*, *Alteromonas vaga*, and *Alteromonas communis* are located in the same rRNA superfamily. (iii) The genus *Beijerinckia* appears to be rather heterogeneous. Its closest relatives appear to be *Xanthobacter autotro-phicus*, "*Mycobacterium*" *flavum*, "*Pseudomonas*" *azotocolligans*, "*Pseudomo-nas*" *diminuta*, the authentic rhodopseudomonads, and some other organisms. These organisms belong in the same rRNA superfamily as *Azospirillum*, *Agro-bacterium*, *Rhizobium*, *Acetobacter*, *Gluconobacter*, and *Zymomonas*. (iv) *Derxia* belongs in still another rRNA superfamily, together with *Chromobacterium*, *Janthinobacterium*, the *Pseudomonas acidovorans* and *Pseudomonas solana-cearum* groups, *Alcaligenes*, and a few other taxa. (v) The following organisms were generically misnamed: "*Azomonas insignis*" ATCC 12523, "*Mycobacte-rium*" *flavum* 301, "*Pseudomonas*" *azotocolligans* ATCC 12417, "*Pseudomonas*" *diminuta* CCEB 513, and "*Rhodopseudomonas*" *gelatinosa* (all strains exam-ined).

Molecular biological methods, such as deoxy-ribonucleic acid (DNA)-DNA or DNA-ribo-somal ribonucleic acid (rRNA) hybridizations, which directly compare bacterial genomes, have opened new perspectives for bacterial classi-fication. Many bacterial genera are phylogenet-ically too far removed from each other to form stable DNA-DNA hybrids. DNA-DNA hybrid-izations are useful either within a genus, such as *Agrobacterium* (20), or between genera which have not diverged too much, such as in the *Enterobacteriaceae* (12; D. Izard, C. Ferragut, and H. Leclerc, in press). rRNA's are conserva-tive molecules (25, 36, 43). There is a good

correlation between the degree of rRNA similar-ity and the overall phenotypic similarity of bac-terial genera (19, 23). Similarity between rRNA cistrons appears to be a good criterion for the classification of bacteria on generic and supra-generic levels. In this paper we attempt to clarify the intra- and intergeneric relationships of sev-eral N₂-fixing bacterial taxa through DNA-rRNA hybridizations.

Until now classification within the family *Azotobacteriaceae* has been based mainly on phenotypic features (13, 31). Only one molecular biological character, namely DNA base compo-sition (guanine plus cytosine [G+C] content),

has been examined (15, 18). In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (13), these characters were used to distinguish four genera in this family. The genus *Azotobacter*, which was established by Beijerinck (7), consists of cyst-forming free-living, aerobic, gram-negative, nitrogen-fixing bacteria with 63 to 66 mol% G+C content. Winogradsky (49) proposed including the *Azotobacter* species that do not form thick-walled cysts in a new genus, *Azomonas*; the G+C values of members of this genus range from 53 to 59 mol%. Starkey and De (41) isolated from Indian rice field soils a nitrogen-fixing organism which they named *Azotobacter indicum*. Because of its morphological and physiological differences from other *Azotobacter* species, Derx (21) proposed including this species in a new genus, *Beijerinckia*; its G+C content ranges from 55 to 61 mol%. The genus *Derxia* was proposed by Jensen et al. (32) for the nitrogen-fixing organism they isolated from West Bengal soil and which appeared different in many features from all previously known nitrogen-fixing strains. They included it in the *Azotobacteriaceae* because of its capacity to fix large amounts of nitrogen. Its G+C content ranges from 69 to 72 mol%. We performed DNA-rRNA hybridizations between labeled reference rRNA's from *Azotobacter chroococcum*, *Azotobacter paspali*, *Azomonas agilis*, *Azomonas insignis*, and *Beijerinckia indica* strains and the DNA of a great variety of bacteria to measure the similarity of their rRNA cistrons and to establish the degree of heterogeneity within the family *Azotobacteriaceae*.

We also performed hybridizations with reference rRNA from *Azospirillum brasilense*. *Azospirillum* have been isolated from the rhizospheres of a variety of grasses, legumes, and grain crops and from soils in tropical and temperate regions; they attracted special attention because of their ability to fix nitrogen (24). The name *Azospirillum* was recently proposed by Tarrand et al. (44). The genus contains two species, *Azospirillum lipoferum* and *Azospirillum brasilense*. We included the type strain (ATCC 29145) of *Azospirillum brasilense* and a reference strain (SpBr17) of *Azospirillum lipoferum* in the present study in order to determine the taxonomic position of *Azospirillum*.

We also examined some other free-living, N_2 -fixing bacteria, such as *Xanthobacter autotrophicus*, "*Mycobacterium*" *flavum*, and some rhodospseudomonads.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Bacteriological purity of the cultures was tested by

plating and by microscopic examination of living and Gram-stained cells. Mass cultures were grown in Roux flasks on media described previously (23). On a solid medium, *Azotobacter chroococcum* NCIB 8002 and NCIB 8003 and *Derxia gummosa* NCIB 9064 produced too much slime, thus preventing harvesting of the cells. These two organisms were grown in liquid culture in broad-bottomed Erlenmeyer flasks; shaking provided good aeration. The *Xanthobacter autotrophicus* and "*Mycobacterium*" *flavum* strains were grown in the laboratory of H. G. Schlegel. We received them as freeze-dried cell powders.

Preparation of high-molecular-weight DNA. DNA samples were prepared by the method described by Marmur (35). They were purified by CsCl gradient centrifugation, denatured, and fixed on cellulose nitrate membrane filters as described previously (17, 23).

Preparation of [^{14}C]rRNA. Radioactively labeled rRNA was prepared as described previously (17, 23), using [2- ^{14}C]uracil as precursor. The specific activities of these rRNA's were as follows: 9,898 and 9,997 cpm/ μ g for the 23S and 16S fractions of *Azotobacter chroococcum* NCIB 8002, respectively; 13,646 and 14,336 cpm/ μ g for the 23S and 16S fractions of *Azotobacter paspali* 8A, respectively; 14,989 and 15,698 cpm/ μ g for the 23S and 16S fractions of *Azomonas agilis* NCIB 8636, respectively; and 6,704 and 6,398 cpm/ μ g for the 23S and 16S fractions of *Azomonas insignis* WR 30, respectively. *Beijerinckia indica* NCIB 8712 and *Azospirillum brasilense* ATCC 29145 did not incorporate [2- ^{14}C]uracil. With [6- ^{14}C]orotate as a precursor for labeling, the rRNA of *B. indica* NCIB 8712 had specific activities of 1,015 and 959 cpm/ μ g for the 23S and 16S fractions, respectively, whereas the rRNA of *Azospirillum brasilense* ATCC 29145 had specific activities of 2,267 and 2,300 cpm/ μ g for the 23S and 16S fractions. *B. indica* NCIB 8712 and *Azospirillum* ATCC 29145 were selected because they are the type strains of their respective species (13, 44), and the four other strains were selected because they were among the oldest available in their taxa. *Azotobacter chroococcum* NCIB 8002 appears to be the original *Azomonas* strain 1 of Winogradsky (9). *Azotobacter paspali* 8A, kindly provided by J. Döbereiner, was one of the original strains of Döbereiner. *Azomonas agilis* NCIB 8636 is one of the strains of J. Smit (9). *Azomonas insignis* WR 30 was strain 8 of V. Jensen and was isolated in 1954 (J. P. Thompson, personal communication).

Hybridizations. Hybridizations, ribonuclease treatments, and thermal stability measurements of the hybrids were performed as described previously (17, 23). Hybridizations were carried out in 2 $\times$ sodium saline citrate buffer (1 $\times$ sodium saline citrate buffer is 0.15 NaCl plus 0.015 M sodium citrate), pH 7.0 containing 20% formamide at the stringent temperature of 50°C (17). For the experiments with the rRNA of *B. indica* NCIB 8712, a slight modification was needed because of the low specific activity; the thermal stability was measured by increasing temperature steps of 10°C instead of 5°C. By counting each sample for 100 min, the results were well within the limits of sensitivity. We used 23S labeled rRNA in all hybridizations except those with *B. indica*, where we had to use 16S.

TABLE 1. List of organisms used, their strain numbers, DNA base compositions and properties of the DNA-rRNA hybrids with three ^{14}C -labeled rRNA references

Sequence no. in Fig. 2 and 3	Species used for DNA prepn	Strain no.	G+C content (mol%) ^a	Hybridization with [^{14}C]rRNA from:					
				<i>Azotobacter</i> <i>chroococcum</i> NCIB 8002		<i>B. indica</i> NCIB 8712		<i>Azospirillum</i> <i>brasiliense</i> ATCC 29145	
				<i>T_m(r)</i> (°C)	rRNA binding (%)	<i>T_m(r)</i> (°C)	rRNA binding (%)	<i>T_m(r)</i> (°C)	rRNA binding (%)
1	<i>Azotobacter chroococcum</i>	NCIB 8002	(65.9)	81	0.167				
2	<i>Azotobacter chroococcum</i>	NCIB 8003	66.2	81	0.160				
3	<i>Azotobacter chroococcum</i>	NCIB 8515	67.5	81	0.166				
4	<i>Azotobacter chroococcum</i>	NCIB 9125	66.0	81	0.140				
5	<i>Azotobacter chroococcum</i>	DSM 281	66.3	81	0.166				
6	<i>Azotobacter chroococcum</i>	DSM 328	66.7	81	0.150				
7	<i>Azotobacter chroococcum</i>	DSM 368	(66.3)	80.5	0.163				
8	<i>Azotobacter chroococcum</i>	DSM 369	(66.1)	81	0.147				
9	<i>Azotobacter chroococcum</i>	DSM 374	65.8	81	0.152				
10	<i>Azotobacter chroococcum</i>	DSM 377	66.4	80.5	0.133	61	0.069		
11	<i>Azotobacter beijerinckii</i>	NCIB 9067	66.2	79.5	0.143				
12	<i>Azotobacter beijerinckii</i>	NCIB 9126	(66.3)	80.5	0.140				
13	<i>Azotobacter beijerinckii</i>	DSM 282	66.0	80	0.161				
14	<i>Azotobacter beijerinckii</i>	DSM 367	66.2	80	0.173				
15	<i>Azotobacter beijerinckii</i>	DSM 373	(65.5)	80	0.141	60.5	0.060		
16	<i>Azotobacter beijerinckii</i>	DSM 378	66.2	80	0.177				
17	<i>Azotobacter beijerinckii</i>	DSM 381	(65.6)	80	0.133				
18	<i>Azotobacter vinelandii</i>	NCIB 8789	66.5	78	0.167				
19	<i>Azotobacter vinelandii</i>	NCIB 9068	(65.6)	77.5	0.145				
20	<i>Azotobacter vinelandii</i>	NCIB 8660	65.0	79	0.147				
21	<i>Azotobacter vinelandii</i>	DSM 85	(65.0)	78.5	0.159	59	0.087		
22	<i>Azotobacter vinelandii</i>	DSM 86	(66.3)	78.5	0.140				
23	<i>Azotobacter vinelandii</i>	DSM 366	64.9	78.5	0.163				
24	<i>Azotobacter vinelandii</i>	DSM 382	(66.3)	79	0.130				
25	<i>Azotobacter vinelandii</i>	DSM 389	(66.8)	79	0.133				
26	<i>Azotobacter vinelandii</i>	C2sm	65.8	78	0.171				
27	<i>Azotobacter miscellum</i>	ATCC 17962	65.6	78.5	0.130				
28	<i>Azotobacter paspali</i>	DSM 88	(63.0)	77.5	0.165				
29	<i>Azotobacter paspali</i>	DSM 376	(63.4)	77.5	0.150				
30	<i>Azotobacter paspali</i>	DSM 383	(64.6)	78.5	0.144				
31	<i>Azotobacter paspali</i>	DSM 388	(63.0)	78	0.153				
32	<i>Azotobacter paspali</i>	DSM 391	63.9	78.5	0.139				
33	<i>Azotobacter paspali</i>	DSM 400	63.8	77.5	0.164	59	0.052		
34	<i>Azotobacter paspali</i>	Döbereiner 8A	63.2	78	0.171				
35	<i>Azotobacter paspali</i>	Döbereiner 15B	63.3	78	0.160				
36	<i>Azotobacter paspali</i>	Döbereiner 22B	63.7	78	0.151				
37	<i>Azotobacter paspali</i>	Döbereiner 23A	64.6	78.5	0.166				
38	<i>Azotobacter nigricans</i>	WR 128	64.5	79	0.161				
39	<i>Azotobacter armeniae</i>	WR 136	65.0	80	0.171				
40	<i>Azotobacter armeniae</i>	WR 138	63.5	79	0.153				
41	<i>Azotobacter armeniae</i>	WR 139	64.7	80	0.167				
42	<i>Azomonas agilis</i>	WR 54	52.9	77	0.106				
43	<i>Azomonas agilis</i>	NCIB 8636	52.6	76	0.088				
44	<i>Azomonas agilis</i>	NCIB 8637	53.2	77	0.087				
45	<i>Azomonas agilis</i>	NCIB 8638	(53.2)	77	0.088				
46	<i>Azomonas agilis</i>	DSM 89	52.0	76	0.092	62	0.066		
47	<i>Azomonas agilis</i>	DSM 375	(51.9)	77	0.100				
48	<i>Azomonas agilis</i>	SS ₁	52.8	76	0.077				
49	<i>Azomonas macrocyto-</i> <i>genes</i>	NCIB 8700	(59.0)	76.5	0.130	58	0.060		
50	<i>Azomonas macrocyto-</i> <i>genes</i>	NCIB 8701	(59.2)	76	0.129				
51	<i>Azomonas macrocyto-</i> <i>genes</i>	NCIB 8702	(59.0)	76	0.136				
52	<i>Azomonas macrocyto-</i> <i>genes</i>	NCIB 9128	58.6	77	0.118				
53	<i>Azomonas macrocyto-</i> <i>genes</i>	NCIB 9129	58.2	76	0.118				
54	" <i>Azomonas insignis</i> " ^{nb}	ATCC 12523	43.4	68	0.139				
55	<i>Azomonas insignis</i>	Tchan C	55.1	76	0.115				

TABLE 1 (continued)

Sequence no. in Fig. 2 and 3	Species used for DNA prepn	Strain no.	G+C content (mol%) ^a	Hybridization with [¹⁴ C]rRNA from:					
				<i>Azotobacter chroococcum</i> NCIB 8002		<i>B. indica</i> NCIB 8712		<i>Azospirillum brasiliense</i> ATCC 29145	
				<i>T_m(_r)</i> (°C)	rRNA binding (%)	<i>T_m(_r)</i> (°C)	rRNA binding (%)	<i>T_m(_r)</i> (°C)	rRNA binding (%)
56	<i>Azomonas insignis</i>	Tchan D	55.6	76.5	0.108	62.5	0.054		
57	<i>Azomonas insignis</i>	WR 12	(58.0)	76	0.107				
58	<i>Azomonas insignis</i>	WR 29	(57.6)	76.5	0.117				
59	<i>Azomonas insignis</i>	WR 30	57.5	77	0.111				
60	<i>Azomonas insignis</i>	WR 31	57.1	77	0.121				
61	<i>Azomonas insignis</i>	WR 56	(57.5)	76.5	0.114				
62	<i>Azomonas insignis</i>	WR 59	57.8	77	0.134				
63	<i>Azomonas insignis</i>	WR 60	(57.5)	77	0.133				
64	<i>Azomonas insignis</i>	WR 61	(57.8)	75.5	0.130				
65	<i>Azomonas insignis</i>	WR 62	58.3	76	0.134				
66	<i>D. gummosa</i>	D	71.4	63	0.061				
67	<i>D. gummosa</i>	D12	72.6	65.5	0.057				
68	<i>D. gummosa</i>	NCIB 9064	69.2	63.5	0.051	55	0.028		
69	<i>B. indica</i>	NCIB 8712	56.4	61.5	0.064	78	0.520		
70	<i>B. indica</i>	NCIB 8005	58.2			78	0.281		
71	<i>B. indica</i>	NCIB 8597	56.3			77	0.267		
72	<i>B. indica</i>	LMD 38.7	57.6			78	0.333		
73	<i>B. indica</i>	(Hilger)	57.4	59	0.051	77.5	0.292		
74	<i>B. fluminensis</i>	(Hilger)	56.2	60	0.067	75.5	0.324		
75	<i>B. derxii</i>	Tchan Q 13	58.5			75.5	0.286		
76	<i>B. laticogenes</i>	NCIB 8846	58.5	60	0.041	75.5	0.301	69	0.047
77	<i>B. mobilis</i>	LMD 50.27	57.3			75	0.137		
78	<i>Xanthobacter autotrophicus</i>	Schlegel 7C	67.3	59	0.034	69.5	0.048	68.5	0.036
79	<i>Xanthobacter autotrophicus</i>	Schlegel GZ29	67.4			71.5	0.044		
80	<i>Xanthobacter autotrophicus</i>	Schlegel JW50	(67.1)			72	0.042	67	0.033
81	<i>Xanthobacter autotrophicus</i>	Schlegel JW33	66.8	60.5	0.028	70.5	0.043		
82	<i>Xanthobacter autotrophicus</i>	Schlegel JW42	64.9	59.5	0.029	70	0.039		
83	" <i>Mycobacterium</i> " <i>flavum</i>	301	68.1			71	0.039	69	0.021
84	<i>Azospirillum brasilense</i>	ATCC 29145	67.5			68	0.121	82.5	0.158
85	<i>Azospirillum brasilense</i>	LMD 50.39	64.6			68	0.131	82	0.169
86	<i>Azospirillum brasilense</i>	Vlassak S11	68.4	63	0.140	68	0.136	82.5	0.165
87	<i>Azospirillum brasilense</i>	Vlassak S19	68.4	63	0.142	66.5	0.120	82	0.154
88	<i>Azospirillum brasilense</i>	Vlassak Si97	66.0			67	0.094	82	0.107
89	<i>Azospirillum brasilense</i>	Vlassak M4	(68.2)			68	0.089	82.5	0.094
90	<i>Azospirillum brasilense</i>	Vlassak S631	(67.9)					82.5	0.161
91	<i>Azospirillum brasilense</i>	Vlassak T2W	(67.9)					82.5	0.144
92	<i>Azospirillum brasilense</i>	Vlassak T2R	(68.2)					82.5	0.157
93	<i>Azospirillum brasilense</i>	Vlassak Sg1	(67.7)					80	0.118
94	<i>Azospirillum brasilense</i>	Vlassak R1	(68.4)					82.5	0.156
95	<i>Azospirillum lipoferum</i>	Döbereiner Sp Br 17	69.0					82	0.125
96	<i>Azospirillum lipoferum</i>	Döbereiner 18c RG	(68.7)					82.5	0.173
97	<i>R. palustris</i>	ATCC 17001	67.2			72	0.042		
98	<i>R. capsulata</i>	ATCC 11166	65.2			69	0.072		
99	<i>R. sphaeroides</i>	ATCC 17023	68.4			69	0.048		
100	<i>R. gelatinosa</i>	ATCC 17011	70.1			59	0.032		
101	<i>R. gelatinosa</i>	Pfennig 2150	71.8			57	0.032		
102	<i>R. gelatinosa</i>	Pfennig 2850	71.8			57.5	0.032		
103	<i>R. gelatinosa</i>	Pfennig Dr2	71.3			58	0.031		
104	<i>R. acidophila</i>	ATCC 25092	64.9			68	0.069		
106	<i>Pseudomonas fluorescens</i>	ATCC 13525	60.2	76.5	0.117				
107	<i>Pseudomonas fluorescens</i>	ATCC 17571		77.5	0.109				
108	<i>Pseudomonas putida</i>	ATCC 12633	62.3	76	0.099				
109	<i>Pseudomonas aeruginosa</i>	CCEB 481	66.8	77	0.094				
110	<i>Pseudomonas synxantha</i>	NCIB 8178	60.4	76	0.097				

TABLE 1 (continued)

Sequence no. in Fig. 2 and 3	Species used for DNA prepn	Strain no.	G+C content (mol%) ^a	Hybridization with [¹⁴ C]rRNA from:					
				Azotobacter chroococcum NCIB 8002		B. indica NCIB 8712		Azospirillum brasiliense ATCC 29145	
				<i>T_m(r)</i> (°C)	rRNA binding (%)	<i>T_m(r)</i> (°C)	rRNA binding (%)	<i>T_m(r)</i> (°C)	rRNA binding (%)
111	<i>Pseudomonas oleovorans</i>	NCTC 10692	(63.5)	77	0.111				
112	<i>Pseudomonas stutzeri</i>	NCTC 10475	64.5	77	0.121				
113	<i>Pseudomonas mucidolens</i>	NCTC 8068	61.0	76	0.099				
114	<i>Pseudomonas acidovorans</i>	ATCC 15668	66.8	64.5	0.099				
115	<i>Pseudomonas desmolytica</i>	ATCC 15005	68.5	65	0.093				
116	<i>Pseudomonas indoloxidans</i>	ATCC 9355 t ₁	66.4	64	0.095				
117	<i>Pseudomonas solanacearum</i>	NCPPB 215	(66.9)	62.5	0.084				
118	<i>Pseudomonas solanacearum</i>	NCPPB 253	(66.8)	64	0.086				
119	" <i>Pseudomonas</i> " azotocoligans	ATCC 12417	65.6	60.5	0.044	70	0.040		
120	" <i>Pseudomonas</i> " diminuta	CCEB 513	67.3			69	0.060		
121	<i>Xanthomonas taraxaci</i>	ICPB T11	64.3	67	0.062				
122	<i>Xanthomonas poinsettiae-color</i>	ICPB P137	66.0	67	0.064				
123	<i>Xanthomonas alfalfae</i>	ICPB A121	67.3	66.5	0.066				
124	<i>Xanthomonas fragariae</i>	NCPPB 1822	63.3	66.5	0.063				
125	" <i>Xanthomonas</i> " ampelina	P7		62	0.072				
126	" <i>Xanthomonas</i> " ampelina	C13	68.2	60.5	0.077				
127	" <i>Xanthomonas</i> " ampelina	2C	68.5	61	0.077				
128	<i>Escherichia coli</i>	B	52.2	69	0.118				
129	<i>Serratia marcescens</i>	NCTC 2847	55.3	68	0.137				
130	<i>Klebsiella rubiacearum</i>	ATCC 15574	59.3	68	0.122				
131	<i>Enterobacter aerogenes</i>	NCTC 10006	53.8	67.5	0.150				
132	<i>Vibrio</i> sp. (noncholera)	E509	49.0	68.5	0.241				
133	<i>Vibrio anguillarum</i>	ATCC 14181	44.2	68	0.198				
134	<i>Vibrio fischeri</i>	NCMB 1281	38.5	66	0.225				
135	<i>Vibrio marinus</i>	NCMB 1143	42.3	66	0.241				
136	<i>Alteromonas haloplanktis</i>	ATCC 19855	41.8	67	0.194				
137	<i>Alteromonas haloplanktis</i>	ATCC 27127	42.6	67	0.161				
138	<i>Alteromonas haloplanktis</i>	ATCC 19648	42.6	68.5	0.208				
139	<i>Alteromonas macleodii</i>	ATCC 27126	45.6	68	0.123				
140	<i>Alteromonas vaga</i>	ATCC 27119	47.7	71.5	0.216				
141	<i>Alteromonas communis</i>	ATCC 27118	47.0	72	0.234				
142	<i>Janthinobacterium lividum</i>	NCTC 9796	65.5	64	0.138				
143	<i>Janthinobacterium lividum</i>	ATCC 14487		63	0.155				
144	<i>Janthinobacterium lividum</i>	MRC RU	65.5	63.5	0.120				
145	<i>Chromobacterium violaceum</i>	NCTC 9757	67.2	64.5	0.171				
146	<i>Chromobacterium violaceum</i>	NCTC 9371	66.1	65.5	0.156				
147	" <i>Chromobacterium</i> " maris-mortui	ATCC 17056	61.8	71.5	0.185				
148	<i>Alcaligenes faecalis</i>	NCIB 8156	57.5	64	0.077				
149	<i>Alcaligenes faecalis</i>	ATCC 19018	58.9	64.5	0.086				
150	<i>Alcaligenes odorans</i>	CCEB 554	56.6	64	0.091				
151	<i>Cellvibrio vulgaris</i>	NCIB 8633	51.5	68	0.069				
152	<i>Microcycylus aquaticus</i>	NCIB 9271		59	0.042				
153	<i>Acetobacter pasteurianus</i>	23 k1 ^a	55.4			66	0.092	68.5	0.121
154	<i>Acetobacter pasteurianus</i>	NCIB 8090	58.0	60.5	0.073				
155	<i>Acetobacter aceti</i>	NCIB 8554	55.9	60.5	0.080				
156	<i>Acetobacter aceti</i>	NCIB 4940	(55.7)			66	0.102		
157	<i>Gluconobacter oxydans</i>	NCIB 8036	62.0			66	0.115	69	0.121
158	<i>Gluconobacter oxydans</i>	116	60.6			66	0.093		

TABLE 1 (continued)

Sequence no. in Fig. 2 and 3	Species used for DNA prepn	Strain no.	G+C content (mol%) ^a	Hybridization with [¹⁴ C]rRNA from:					
				Azotobacter chroococcum NCIB 8002		B. indica NCIB 8712		Azospirillum brasilense ATCC 29145	
				<i>T_{m(e)}</i> (°C)	rRNA binding (%)	<i>T_{m(e)}</i> (°C)	rRNA binding (%)	<i>T_{m(e)}</i> (°C)	rRNA binding (%)
159	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	ATCC 29191	48.8			66	0.080		
160	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	B70				66	0.080		
161	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	5.3	49.3			66	0.070		
162	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	CP3				67	0.090		
163	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	AG11				67	0.090		
164	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	Delft 6				66.5	0.110		
165	<i>Zymomonas mobilis</i> subsp. <i>pomaceae</i>	ATCC 29192	47.7			66.5	0.080		
166	<i>Agrobacterium tumefaciens</i>	ICPB TT111	60.6			67	0.074	65	0.042
167	<i>Agrobacterium tumefaciens</i>	CIP B6	61.8			67	0.059		
168	" <i>Agrobacterium</i> " <i>aggregatum</i>	Ahrens B1	58.7			72	0.063		
169	" <i>Agrobacterium</i> " <i>gelatinovorum</i>	Ahrens B6	57.6			67.5	0.029		
170	" <i>Agrobacterium</i> " <i>luteum</i>	Ahrens B14	52.5			66	0.033		
171	" <i>Azotomonas</i> " <i>insolita</i>	ATCC 12412	60.5			68	0.071		
172	" <i>Azotomonas</i> " <i>species</i>	ATCC 12210	(58.0)			67.5	0.058		
173	" <i>Chromobacterium</i> " <i>folium</i>	NCTC 10591	63.0			66.5	0.045		
174	<i>Paracoccus denitrificans</i>	ATCC 19367	67.4			67	0.049	67.5	0.061
175	<i>Paracoccus denitrificans</i>	ATCC 17741	66.4			66	0.060		
176	<i>S. itersonii</i> subsp. <i>vulgatum</i>	NCIB 9071	62.3			69	0.088	72.5	0.111
177	<i>S. polymorphum</i>	NCIB 9072	63.7					71.5	0.075

^a The G+C contents were determined in our laboratory by thermal denaturation, except for those taxa with values in parentheses, which were determined from absorbance ratios (14).

^b Organisms with taxon names in quotation marks are misnamed and do not belong in that taxon.

RESULTS

Sucrose gradient centrifugation of rRNA.

An example of a sucrose gradient sedimentation pattern for each of the six reference rRNA's is shown in Fig. 1. The 23S fraction of *B. indica* NCIB 8712 was much smaller than its 16S fraction. The same kind of profile was found for *Agrobacterium tumefaciens* TT111 and *Agrobacterium rhizogenes* TR7 (23) and for *Acetobacter aceti* NCIB 8621 (M. Gillis and J. De Ley, manuscript in preparation). We show below that *Beijerinckia* appears to be a relative of these taxa.

DNA-rRNA hybridizations. The results of the hybridizations are expressed as the temperature at which 50% of a hybrid was denatured (*T_{m(e)}*) and the percentage of rRNA binding (micrograms of ¹⁴C-labeled rRNA duplexed per

100 µg of filter-fixed DNA). By plotting *T_{m(e)}* against percent binding, we obtained rRNA similarity maps. It has to be stressed that the percent rRNA binding is not a measure of rRNA homology, because the latter also depends on the number of rRNA cistrons per genome and the size and the state of replication of the genome (23). It has been reported previously (19, 23) that there is a good correlation between the degree of rRNA similarity, expressed as *T_{m(e)}*, and the overall phenotypic similarity of bacterial genera or subgenera. Each taxon occupies a definite area on the rRNA similarity map. The size and shape of this area depend on the phenotypic and genetic heterogeneity of the taxon when many strains are included. A few strains suffice to locate a taxon area on the map. Table 2 shows the *T_{m(e)}* values and the percentages of rRNA binding of the reciprocal hybrids between the

FIG. 1. Fractionation of ^{14}C -labeled rRNA's on 15 to 30% linear sucrose gradients. The method used has been described previously (17).

DNAs of *Azotobacter*, *Azomonas*, *Beijerinckia*, and *Derxia* strains and ^{14}C -labeled rRNA from representative strains of various other genera. $T_{m(e)}$ values of the reciprocal hybrids for each set of two strains were almost the same.

Similarity of *Azotobacter* rRNA cistrons. The rRNA similarity map of *Azotobacter chroococcum* NCIB 8002 is shown in Fig. 2. The rRNA cistrons of all *Azotobacter* strains were highly similar ($T_{m(e)}$, from 77.5 to 81°C). Although the differences in $T_{m(e)}$ were small, the species that produce a water-soluble fluorescent pigment (*Azotobacter vinelandii* and *Azotobacter paspali*, with $T_{m(e)}$ values of 77.5 to 79°C) could be differentiated from the species that do not produce such a pigment (*Azotobacter chroococcum*, *Azotobacter beijerinckii*, *Azotobacter nigricans*, and *Azotobacter armeniae*, with $T_{m(e)}$ values between 79 and 81°C). This differentiation was also found with [^{14}C]rRNA from *Azotobacter paspali* 8A (Table 3); *Azotobacter paspali* and *Azotobacter vinelandii* strains had $T_{m(e)}$

values of 81 to 81.5°C, whereas the remaining *Azotobacter* strains had $T_{m(e)}$ values ranging from 77.5 to 79°C.

Except for *Azomonas insignis* ATCC 12523, the DNA-rRNA hybrids of the *Azomonas* strains had high $T_{m(e)}$ values (75.5 to 77°C). The $T_{m(e)}$ of 68°C for strain ATCC 12523 was far outside the range of *Azomonas*. We received two subcultures of this strain from the American Type Culture Collection, and both gave the same results. This strain is discussed below.

The strains belonging to *Pseudomonas* section I in *Bergey's Manual of Determinative Bacteriology* (13) grouped at a high $T_{m(e)}$ (76 to 77°C). These strains were *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas fluorescens* ATCC 17571, *Pseudomonas putida* ATCC 12633, *Pseudomonas aeruginosa* CCEB 481, *Pseudomonas synxantha* NCIB 8178, *Pseudomonas oleovorans* NCTC 10692, *Pseudomonas stutzeri* NCTC 10475, and *Pseudomonas mucidolens* NCTC 8068. The close relationship of

TABLE 2. Hybridizations between DNAs of a few *Azotobacter*, *Azomonas*, *Beijerinckia* and *Derrxia* strains and ¹⁴C-labeled rRNA's from various reference strains^a

Hybridization with [¹⁴ C]rRNA from:												
DNA from	<i>Pseudomonas fluorescens</i> ATCC 13525	<i>Escherichia coli</i> B	<i>Xanthomonas campestris</i> NCPPB 528	<i>Pseudomonas acidovorans</i> ATCC 15668	<i>Pseudomonas solanaceae</i> NCPPB 325	<i>Alcaligenes faecalis</i> NCIB 8156	<i>Chromobacterium violaceum</i> NCTC 9757	<i>Janthinobacterium lividum</i> NCTC 9796	<i>Agrobacterium tumefaciens</i> ICPB TT111			
	<i>T_m(_{ic})</i> (°C)	% rRNA binding	<i>T_m(_{ic})</i> (°C)	% rRNA binding	<i>T_m(_{ic})</i> (°C)	% rRNA binding	<i>T_m(_{ic})</i> (°C)	% rRNA binding	<i>T_m(_{ic})</i> (°C)	% rRNA binding	% rRNA binding	
<i>Azotobacter vinelandii</i> NCIB 8660	75	0.10	67	0.13					62	0.12	62	0.07
<i>Azotobacter paspali</i> Döbereiner 22B	76	0.14	67.5	0.14	67	0.13					59.5	0.09
<i>Azotobacter paspali</i> Döbereiner 15B					66	0.12			61	0.10		
<i>Azotobacter chroococcum</i> DSM 281	75	0.12			68.5	0.12						
<i>Azotobacter chroococcum</i> DSM 369					67.5	0.12						
<i>Azotobacter beijerinckii</i> DSM 367	75	0.13										
<i>Azotobacter vinelandii</i> DSM 86	75.5	0.12										
<i>Azotobacter miscellum</i> ATCC 17962					67.5	0.10						
<i>Azomonas agilis</i> SS4	76	0.10	66	0.07					62	0.06	63.5	0.08
<i>Azomonas agilis</i> DSM 89					65.5	0.06						
<i>Azomonas macrocytogenes</i> NCIB 8700	76.5	0.13	67	0.07							61.5	0.07
<i>B. indica</i> (Hilger)	60.5	0.07			54	0.02						
<i>B. fluminensis</i> (Hilger)	59.5	0.09			57.5	0.04					67.5	0.09
<i>D. gummosa</i> NCIB 9064											67	0.12
<i>D. gummosa</i> D	63	0.09			69	0.05			70	0.06		
<i>D. gummosa</i> D12	62	0.07			69	0.04			70.5	0.06		
					71	0.06					71	0.09

^aThe *Agrobacterium*, *Chromobacterium*, and *Janthinobacterium* data are from references 19 and 23. All other data are unpublished from J. De Ley, P. De Vos, J. De Smedt, R. Tytgat, and P. Segers (manuscripts in preparation).

FIG. 2. Similarity map of hybrids between the 23S ^{14}C -labeled rRNA fraction of *Azotobacter chroococcum* NCIB 8002 and the DNAs from a variety of bacteria. $T_{m(e)}$ and percent rRNA binding were as defined in the text. The positions of the organisms belonging to the family Azotobacteriaceae of Bergey's Manual, 8th ed. (13), are indicated by their sequence numbers (Table 1); the positions of the other strains are indicated by symbols (x, +, o, and ●). Strains belonging phenotypically to the same taxon are surrounded by a closed line. These areas locate the taxa on the map; their shapes and dimensions are limited by the number of strains used, and the line is not the ultimate border.

the rRNA cistrons of these organisms to those of *Azotobacter* and *Azomonas* was confirmed by reverse hybridizations with labeled rRNA from *Pseudomonas fluorescens* ATCC 13525 (Table 2).

The *Xanthomonas* species were close together on the rRNA similarity map, with a $T_{m(e)}$ of 67°C and 0.065% binding, except for the *Xanthomonas ampelina* strains, which had a $T_{m(e)}$ of 60.5 to 62°C. Reverse hybridizations with reference rRNA from *Xanthomonas campestris* NCPPB 528 have shown that the rRNA cistrons of "*Xanthomonas*" *ampelina* strains are quite different from those of authentic *Xanthomonas* species (P. De Vos and J. De Ley, manuscript in preparation).

The $T_{m(e)}$ of the DNA-rRNA hybrid of "*Chromobacterium*" *maris-mortui* ATCC 17059 was

71.5°C. As described previously (19), DNA-rRNA hybridizations have shown that this strain does not belong in either *Chromobacterium* or *Janthinobacterium*. From our results we can conclude that there is a remote relationship with *Azotobacter* and *Pseudomonas* section I (13), although ATCC 17059 does not belong in either of these taxa.

Representative strains of the *Enterobacteriaceae* were grouped at 67.5 to 69°C and 0.12 to 0.15% rRNA binding, whereas strains of the *Vibrionaceae* were grouped at 66 to 68.5°C and 0.20 to 0.24% rRNA binding; *Cellvibrio vulgaris* NCIB 8633 was at about the same distance ($T_{m(e)}$ of 68°C and 0.07% rRNA binding). The *Alteromonas* species were heterogeneous; *Alteromonas haloplanktis* and *Alteromonas macleodii* had $T_{m(e)}$ values of 67 to 68.5°C, whereas

Alteromonas vaga and *Alteromonas communis* had $T_{m(e)}$ values of 71.5 and 72°C, respectively.

Several taxa were located on the rRNA similarity map between $T_{m(e)}$ values of 59 and 65°C; these included *Chromobacterium*, *Janthinobacterium*, the authentic *Alcaligenes* strains, *Pseudomonas* strains of section III in *Bergey's Manual* (13), *Derxia*, *Beijerinckia*, *Azospirillum*, *Acetobacter*, *Xanthobacter autotrophicus*, *Microcylus*, and "*Xanthomonas*" *ampelina*. A $T_{m(e)}$ of 65°C seems to be the lower limit for significant taxonomic conclusions (19, 23; J. De Ley et al., manuscript in preparation).

Similarity of *Azomonas* rRNA cistrons. Hybridizations were also performed between the DNAs of all of our *Azomonas* strains and reference [14 C]rRNA from *Azomonas insignis* WR 30 and *Azomonas agilis* NCIB 8636. DNAs of some *Azotobacter* strains were also included. The results are shown in Table 3. Obviously, all of these nitrogen-fixing bacteria are closely related. Nevertheless, the rRNA cistrons of *Azomonas agilis*, *Azomonas insignis*, and *Azomonas macrocytogenes* differ as much from each other as from the RNA cistrons of azotobacters.

Similarity of *Beijerinckia* rRNA cistrons.

TABLE 3. Properties of the hybrids between DNAs from *Azomonas* and *Azotobacter* strains and 14 C-labeled rRNA's from *Azomonas agilis* NCIB 8636, *Azomonas insignis* WR 30, and *Azotobacter paspali* 8A

Species used for DNA prepn	Strain no.	Hybridization with [14 C]rRNA from:					
		<i>Azomonas agilis</i> NCIB 8636		<i>Azomonas insignis</i> WR 30		<i>Azotobacter paspali</i> 8A	
		$T_{m(e)}$ (°C)	% rRNA Binding	$T_{m(e)}$ (°C)	% rRNA Binding	$T_{m(e)}$ (°C)	% rRNA Binding
<i>Azomonas agilis</i>	NCIB 8636	79	0.164	76.5	0.102		
<i>Azomonas agilis</i>	NCIB 8637	79	0.153	76.5	0.104	76	0.078
<i>Azomonas agilis</i>	NCIB 8638	79	0.162	76	0.104		
<i>Azomonas agilis</i>	DSM 375	78	0.132	76.5	0.112		
<i>Azomonas agilis</i>	DSM 89	78	0.160	75.5	0.114		
<i>Azomonas agilis</i>	SS4	78	0.149	76	0.115		
<i>Azomonas agilis</i>	WR 54	78.5	0.134	77	0.102		
<i>Azomonas macrocytogenes</i>	NCIB 9128	76.5	0.118	76.5	0.109	77	0.096
<i>Azomonas macrocytogenes</i>	NCIB 9129	75.5	0.112	77	0.101		
<i>Azomonas macrocytogenes</i>	NCIB 8700	76.5	0.148	76.5	0.112		
<i>Azomonas macrocytogenes</i>	NCIB 8701	76.5	0.123	76.5	0.121	76.5	0.101
<i>Azomonas macrocytogenes</i>	NCIB 8702	76	0.133	77	0.138		
<i>Azomonas insignis</i>	WR 30	76.5	0.124	80.5	0.139	76.5	0.099
<i>Azomonas insignis</i>	WR 12	76	0.123	80	0.142		
<i>Azomonas insignis</i>	WR 29	76	0.120	80.5	0.136		
<i>Azomonas insignis</i>	WR 31	75.5	0.132	80	0.143		
<i>Azomonas insignis</i>	WR 56	75.5	0.120	80	0.126		
<i>Azomonas insignis</i>	WR 59	76	0.136	80.5	0.159		
<i>Azomonas insignis</i>	WR 60	76	0.134	80.5	0.164		
<i>Azomonas insignis</i>	WR 61	75	0.139	78	0.159		
<i>Azomonas insignis</i>	WR 62	75.5	0.131	79.5	0.162		
<i>Azomonas insignis</i>	Tchan C	76	0.123	80.5	0.138		
<i>Azomonas insignis</i>	Tchan D	76	0.118	80.5	0.122		
<i>Azotobacter chroococcum</i>	NCIB 8515	76.5	0.137	76.5	0.134		
<i>Azotobacter chroococcum</i>	DSM 328	76	0.136	76.5	0.150	78	0.104
<i>Azotobacter beijerinckii</i>	DSM 282	76	0.138	76	0.152		
<i>Azotobacter beijerinckii</i>	NCIB 9067					77.5	0.109
<i>Azotobacter beijerinckii</i>	NCIB 9126					78	0.098
<i>Azotobacter vinelandii</i>	NCIB 8660	75	0.142	75	0.144		
<i>Azotobacter vinelandii</i>	NCIB 9068					81	0.123
<i>Azotobacter vinelandii</i>	DSM 86					81.5	0.114
<i>Azotobacter vinelandii</i>	DSM 366					81	0.123
<i>Azotobacter vinelandii</i>	DSM 382					81	0.110
<i>Azotobacter vinelandii</i>	DSM 389					81.5	0.103
<i>Azotobacter paspali</i>	Döbereiner 8A					81.5	0.149
<i>Azotobacter paspali</i>	Döbereiner 23A	76	0.149	75	0.167	81.5	0.140
<i>Azotobacter paspali</i>	DSM 88					81.5	0.167
<i>Azotobacter paspali</i>	DSM 376					81.5	0.139
<i>Azotobacter paspali</i>	DSM 383					81.5	0.132
<i>Azotobacter paspali</i>	DSM 391					81.5	0.139
<i>Azotobacter armeniae</i>	WR 136	75	0.148	75.5	0.154		
<i>Azotobacter armeniae</i>	WR 138					78.5	0.103
<i>Azotobacter armeniae</i>	WR 128					79	0.124
" <i>Azomonas insignis</i> "	ATCC 12523	68.5	0.126	69.5	0.130		

The rRNA similarity map of *B. indica* NCIB 8712 is shown in Fig. 3. The $T_{m(e)}$ values of the rRNA cistrons of *Beijerinckia* strains were rather similar to each other (75 to 78°C), but the percent rRNA binding was heterogeneous. The *B. indica* strains were very similar, with $T_{m(e)}$ values of 77 to 78°C and 0.3% binding, except for the homologous duplex with 0.52% binding. The homologous reaction was performed several times with separate DNA preparations and separate DNA filters, always resulting in the same high percent rRNA binding. The $T_{m(e)}$ values of *Beijerinckia lacticoenes* NCIB 8846 and *Beijerinckia fluminensis* (both 75.5°C) differed only slightly from those of *B. indica* strains. *Beijerinckia mobilis* LMD 50.27 was at the same $T_{m(e)}$ level (75°C) but differed clearly from the other *Beijerinckia* strains by its low percent rRNA binding (0.137%). This hybridization was repeated with separate strain LMD 50.27 DNA preparations and separate DNA filters; the same low percent rRNA binding was found in each case.

Several taxa had $T_{m(e)}$ values between 68 and 72°C and percent rRNA binding between 0.03

and 0.07; these included *Rhodopseudomonas palustris*, *Rhodopseudomonas capsulata*, *Rhodopseudomonas sphaeroides*, *Rhodopseudomonas acidophila*, *Spirillum itersonii*, *Xanthobacter autotrophicus*, and the misnamed "*Mycobacterium*" *flavum*, "*Agrobacterium aggregatum*" (23), "*Pseudomonas*" *azotocolligans*, and "*Pseudomonas*" *diminuta*. Another group of taxa was located close to these strains, with $T_{m(e)}$ values from 66 to 68°C; these included *Agrobacterium*, *Acetobacter*, *Gluconobacter*, *Zymomonas*, *Paracoccus*, *Azospirillum*, and the misnamed "*Agrobacterium gelatinovorum*," "*Agrobacterium luteum*," "*Azotomonas insolita*," *Azotomonas* sp. ATCC 12210, and "*Chromobacterium folium*" (23). These results agree very well with the inverse hybridizations between *Beijerinckia* and *Agrobacterium* reported previously (23).

Azotobacter and *Azomonas* strains grouped together, with $T_{m(e)}$ values of 58 to 62°C, whereas *Derxia gummosa* NCIB 9064 had a $T_{m(e)}$ of 55°C. The considerable differences between the rRNA cistrons of *Beijerinckia* and *Azotobacter*-*Azomonas* are obvious from both rRNA similarity

FIG. 3. Similarity map of hybrids between the 16S ^{14}C -labeled rRNA fraction of *B. indica* NCIB 8712 and the DNAs from a variety of bacteria. For additional explanation, see legend to Fig. 2.

maps (Fig. 2 and 3). The *Rhodopseudomonas gelatinosa* strains grouped together, with $T_{m(e)}$ values of 57 to 59°C.

Similarity of *Derxia* rRNA cistrons. The rRNA cistrons of *Derxia* are quite different from those of *Beijerinckia*, *Azotobacter*, and *Azomonas*. The hybrids of *D. gummosa* DNA with rRNA of *Azotobacter chroococcum* NCIB 8002 had the same $T_{m(e)}$ values as the hybrids of strains of *Alcaligenes*, *Chromobacterium*, *Janthinobacterium* (19), and *Pseudomonas* section III of *Bergey's Manual* (Table 1). Therefore, hybridizations were performed between the DNA of *D. gummosa* and reference [^{14}C]rRNA's of these taxa (Table 2). The results proved that *Derxia* is a member of this group of taxa but has a separate position among them ($T_{m(e)}$, approximately 70°C).

Similarity of *Azospirillum* rRNA cistrons. The results of the hybridizations with [^{14}C]rRNA from *Azospirillum brasilense* ATCC 29145 are summarized in Table 1. The rRNA cistrons of all *Azospirillum* strains were very similar ($T_{m(e)}$ values between 80 and 82.5°C). *Spirillum polymorphum* NCIB 9072 and *S. iberonii* subsp. *vulgatum* NCIB 9071 had $T_{m(e)}$ values of 71.5 and 72.5°C, respectively. Representatives of *Paracoccus*, *Agrobacterium*, *Acetobacter*, *Gluconobacter*, *Beijerinckia*, *Xanthobacter autotrophicus*, and "*Mycobacterium*" *flavum* had $T_{m(e)}$ values between 65 and 69°C, showing that *Azospirillum* belongs in the rRNA superfamily of these taxa.

DISCUSSION

The taxonomic relationships among numerous genera and subgenera of gram-negative bacteria, involving hundreds of bacterial species, are being investigated in our laboratory by DNA-rRNA hybridizations (17, 19, 23; De Ley et al., manuscript in preparation). With this method we have been able to classify these taxa into several rRNA superfamilies. The first rRNA superfamily contains the *Enterobacteriaceae* and the *Vibrionaceae*; the second superfamily consists of *Pseudomonas* section I of *Bergey's Manual* (13), *Xanthomonas*, *Aplanobacter*, and several other taxa; the third rRNA superfamily consists of *Chromobacterium*, *Janthinobacterium* (19), *Pseudomonas* sections II and III (13), the authentic *Alcaligenes* strains, and several other taxa; and the fourth rRNA superfamily consists mainly of taxa connected with the phytosphere, i.e. *Rhizobium*, *Agrobacterium*, *Phyllobacterium*, *Zymomonas*, *Acetobacter*, *Gluconobacter*, and several other taxa. An important point for the present discussion is that there is a distinct correlation between rRNA similarities, ex-

pressed as $T_{m(e)}$, and the overall phenotypic similarities of the organisms (19, 23; De Ley et al., manuscript in preparation).

It is very striking that 10 strains of *Azotobacter chroococcum*, 7 strains of *Azotobacter beijerinckii*, 9 strains of *Azotobacter vinelandii*, 1 strain of *Azotobacter miscellum* (very likely identical with *Azotobacter vinelandii* [15]), 1 strain of *Azotobacter nigricans*, and 10 strains of *Azotobacter paspali* have rRNA cistrons which are almost identical (Tables 1 and 3) and are thus located very close together on the rRNA similarity map (Fig. 2). It is very encouraging to note that all of the cyst-forming organisms, which are phenotypically very similar, have very similar rRNA cistrons. Our results confirm that the genus *Azotobacter*, as described in *Bergey's Manual*, 8th ed. (13), is a real biological taxon and not a taxonomic artifact. Strains WR 136, WR 138 and WR 139 were isolated from soil in Armenia and were originally classified as *Azotobacter agilis* subsp. *armeniae* (34). In a numerical analysis of the *Azotobacteriaceae*, J. P. Thompson (Ph.D. thesis, University of Queensland, Queensland, Australia, 1975) classified *Azotobacter nigricans* and *Azotobacter armeniae* as separate species in *Azotobacter* and designated *Azotobacter miscellum* a subjective synonym of *Azotobacter vinelandii*. The same author proposed that a new genus be established for *Azotobacter paspali*. However, the latter proposal is opposed by our observations on rRNA similarities, which very strongly suggest that *Azotobacter paspali* is a normal member of the genus *Azotobacter*.

A total of 7 strains of *Azomonas agilis*, 5 strains of *Azomonas macrocytogenes*, and 11 strains of *Azomonas insignis* all fall in the same area on the rRNA similarity map. It should be noted that *Azomonas insignis* ATCC 12523 is misidentified. We received two subcultures of this strain from the American Type Culture Collection, and both gave the same results, as follows. The $T_{m(e)}$ of 68°C is far outside the range of the $T_{m(e)}$ values of authentic *Azomonas insignis* strains. The G+C content of the DNA of ATCC 12523 is 43.4 instead of 55 to 58 mol%. On our growth media, this organism was not motile, contrary to the findings of Derx (22), who described the motility of *Azomonas insignis* as very characteristic. The actual taxonomic position of ATCC 12523 is unknown. De Ley and Park (18) discovered that the DNA base composition of *Azomonas agilis* (51 to 53 mol% G+C) is quite different from that of *Azomonas macrocytogenes* and *Azomonas insignis* (55 to 59 mol% G+C). Therefore, these authors suggested locating *Azomonas agilis* in a separate genus as *Azotococcus agilis* and locating *Azo-*

monas insignis and *Azomonas macrocytogenes* in *Azomonas*. In *Bergey's Manual*, 8th ed. (13), all three species are included in the genus *Azomonas*. We reexamined this problem by hybridizing DNAs from organisms in this group with [¹⁴C] rRNA's from *Azomonas insignis* WR 30 and *Azomonas agilis* NCIB 8636 (Table 3). The rRNA cistrons of *Azomonas macrocytogenes*, *Azomonas insignis*, and *Azomonas agilis* differ as much from each other as they do from the rRNA cistrons of azotobacters ($T_{m(e)}$ of the DNA-rRNA hybrids, from 75 to 76.5°C). The relationship between these four taxa can be expressed nomenclaturally in several ways. One solution would be to create three separate genera, one each for *Azomonas agilis*, *Azomonas macrocytogenes*, and *Azomonas insignis*; this might be the most logical solution but it is certainly not the most practical solution for the moment. However, pending further research on phenotypic analysis and genome comparisons, we prefer temporarily to retain the three species in the genus *Azomonas*. This genus is more heterogeneous (G+C content, from 52 to 59 mol%; $T_{m(e)}$ of the intragenomic DNA-rRNA hybrids from 75 to 80.5°C) than is *Azotobacter* (63 to 67.5 mol% G+C; intragenomic $T_{m(e)}$, from 77.5 to 81°C).

Tables 1 and 3 and Fig. 2 show that there is a close similarity in $T_{m(e)}$ values among the rRNA cistrons of azotobacters, the three species of *Azomonas*, and the species of *Pseudomonas* section I (13). In addition, these five taxa are equidistant from each other at a $T_{m(e)}$ of about 76°C. We concluded from these rRNA similarities that the taxa *Azotobacter*, *Azomonas*, and *Pseudomonas* section I belong in the same rRNA superfamily. Our conclusion on the close genetic relatedness between *Pseudomonas* section I and *Azotobacter* is emphasized by a comparison of the structure of another gene product. Ambler (1) found close similarities between the amino acid sequences of cytochrome c-551 of five *Pseudomonas* species from section I and cytochrome c-551 of *Azotobacter vinelandii*. The sequence differences between the cytochromes from the *Pseudomonas* strains were in the range of 20 to 40%, and the cytochrome of *Azotobacter vinelandii* differed by the same amount from the *Pseudomonas* cytochromes. Ambler (1) writes, "It is interesting to note that by this single genetic criterion *A. vinelandii* is as good a *Pseudomonas* as any of the others." The full importance of this observation has been underestimated up to now. However, in conjunction with our results on rRNA cistron similarities, both arguments are powerful evidence that *Pseudomonas*, *Azotobacter*, and *Azomonas* are close branches of the same phylogenetic tree.

The similarity in $T_{m(e)}$ values between the rRNA cistrons of *Azotobacter* and *Azomonas* and the rRNA cistrons of pseudomonads other than the pseudomonads in section I is much smaller ($T_{m(e)}$, 62 to 64°C). The explanation is that the present genus *Pseudomonas* is extremely heterogeneous (36) and consists very likely of at least two genera and a number of misnamed strains (De Vos and De Ley, manuscript in preparation).

The genus *Alteromonas* is heterogeneous (Table 1 and Fig. 2). *Alteromonas vaga* and *Alteromonas communis* are located in the same rRNA superfamily as *Azotobacter* and *Azomonas* at $T_{m(e)}$ values of 71.5 to 72°C. *Alteromonas haloplanktis* and *Alteromonas macleodii* are located in the vicinity of the *Enterobacteriaceae* and the *Vibrionaceae*, with $T_{m(e)}$ values of 67 to 68.5°C. The rRNA heterogeneity of *Alteromonas* agrees very well with the results of a numerical analysis of the phenotypic features of a diversity of aerobic marine bacteria. *Alteromonas vaga* and *Alteromonas communis* grouped together at 72% phenotypic similarity in one group of taxa, whereas *Alteromonas haloplanktis* and *Alteromonas macleodii* clustered at 72% similarity with a second group of taxa; both groups linked at 40% phenotypic similarity (5).

The genus *Beijerinckia* is much more heterogeneous than *Azotobacter* and *Azomonas*. Although the $T_{m(e)}$ values of all of the strains used are limited within a 3°C range, the percent rRNA binding varies from 0.14% for *B. mobilis* LMD 50.27 to 0.52% for the reference strain *B. indica* NCIB 8712 (Table 1 and Fig. 3). The rRNA features of the remaining strains of *B. indica*, *B. lacticogenes*, *B. fluminensis*, and *Beijerinckia derxii* are very similar, with $T_{m(e)}$ values of $76.75 \pm 1.25^\circ\text{C}$ and rRNA binding of $0.30 \pm 0.04\%$. In *Bergey's Manual* (13), *Azotobacter lacticogenes* was identified with *B. indica*, whereas *B. fluminensis*, *B. derxii*, and *B. mobilis* were recognized as separate species. Since *B. lacticogenes* is located in the same region as *B. fluminensis* and *B. derxii* on the rRNA similarity map (Fig. 3), further conclusions on the intragenomic taxonomic relationships of *Beijerinckia* can only be drawn when more phenotypic and genome comparisons become available. The extreme position of *B. mobilis* LMD 50.27 on the rRNA similarity map suggests that it may be a rather exceptional member of the genus. This is corroborated by the unusual phenotypic features of the species *B. mobilis* summarized in Table 7.9 *Bergey's Manual* (13). On the rRNA similarity map (Fig. 3) there are no genera in the immediate vicinity of *Beijerinckia*, which suggests that this genus has a separate phylogenetic

position; it is indeed phenotypically easily distinguished from almost all other genera of bacteria. From Table 1 and Fig. 3, it is at once obvious that rRNA cistrons of *Beijerinckia* are not the closest relatives of those of *Azotobacter*, *Azomonas*, and *Derrxia*. *Beijerinckia* belongs in an rRNA superfamily with *Agrobacterium*, *Rhizobium* (23), *Acetobacter*, *Gluconobacter*, *Zymomonas* (Gillis and De Ley, manuscript in preparation), and other taxa (this paper). The phenotypic similarity among *Agrobacterium*, *Rhizobium*, and *Beijerinckia* has been determined (28). De Ley et al. (16) clustered these three genera on the basis of their average inter- and intra group phenotypic similarities. When strains of these genera were clustered on the basis of their average $T_{m(e)}$ values, it is very striking that a similar dendrogram was obtained (Fig. 4). The rRNA cistrons of *Beijerinckia* most closely resemble those of *Rhodopseudomonas palustris*, *R. sphaeroides*, *R. capsulata*, *Spirillum itersonii* subsp. *vulgatum*, *Xanthobacter autotrophicus*, and the misnamed "*Mycobacterium*" *flavum*, "*Pseudomonas*" *azotocolligans*, "*Pseudomonas*" *diminuta*, and "*Agrobacterium*" *aggregatum* (23).

Schlegel and co-workers (6, 27, 40, 45) isolated

yellow-pigmented, nitrogen-fixing hydrogen bacteria with several properties characteristic of coryneform bacteria and initially classified them as *Corynebacterium autotrophicum* (6). These organisms do not utilize carbohydrates, except gluconate and sometimes fructose, which are catabolized by way of the Entner-Doudoroff pathway. They grow well on organic acids. Recently, Schlegel and co-workers (47) concluded from cell wall analysis and physiological data that this organism is not a coryneform, and they renamed it *Xanthobacter autotrophicus* (48). This is in excellent agreement with our results. Observations in our laboratory (J. De Smedt and J. De Ley, manuscript in preparation) have shown that the rRNA cistrons of true coryneforms are quite different from those of all gram-negative bacteria tested. It is obvious from the position of *Xanthobacter* on the rRNA similarity map (Table 1 and Fig. 3) that it belongs in our fourth rRNA superfamily of gram-negative taxa (see above), in which the presence of the Entner-Doudoroff pathway is a common feature (33).

The yellow-pigmented, nitrogen-fixing "*Mycobacterium*" *flavum* 301 was isolated by Fedorov and Kalininskaya (26) from Russian turf

FIG. 4. Comparison of the phenetic similarity and the similarity of the rRNA cistrons of *Agrobacterium*, *Rhizobium*, and *Beijerinckia*. (a) Phenetic similarity as calculated by De Ley et al. (16) from the data of Graham (28), involving 100 features of 8 strains of *Beijerinckia*, 32 strains of *Rhizobium leguminosarum*, 25 strains of *Rhizobium meliloti*, 27 strains of *Rhizobium japonicum*, and 18 strains of *Agrobacterium tumefaciens* and *Agrobacterium radiobacter*. Each group is represented by an inverted triangle, the bottom tip of which indicates the intragroup mean percent similarity. (b) Linkage of *Agrobacterium*, *Rhizobium*, and *Beijerinckia* according to the $T_{m(e)}$ values of their DNA-rRNA hybrids. $T_{m(e)}$ is expressed in degrees centigrade. The $T_{m(e)}$ values are summarized from De Smedt and De Ley (23) and from the present paper.

podzol soil. This organism does not utilize carbohydrates but grows on ethanol and organic acids. Biggins and Postgate (8) examined a number of features of this strain. Its exact taxonomic position is unknown. "*Mycobacterium*" *flavum* 301 has the same position as *Xanthobacter autotrophicus* on the rRNA similarity map of Fig. 3. This finding points to a possible close relationship between these two taxa. It also shows that "*Mycobacterium*" *flavum* 301 does not belong in *Mycobacterium*, which is completely outside our fourth rRNA superfamily (De Smedt and De Ley, manuscript in preparation).

R. palustris, *R. sphaeroides*, *R. capsulata*, and *R. acidophila* are located in the same region as *Xanthobacter autotrophicus* on the rRNA similarity map compared with *B. indica* NCIB 8712 (Fig. 3). Upon close inspection, some phenotypic similarities between the two genera are evident. Van Niel (46) described the cell morphology of *R. palustris* in older cultures as "strikingly reminiscent of *Corynebacterium* and *Mycobacterium species*." Likewise, *R. capsulata* and *R. sphaeroides* have irregular cell shapes (46). Cell morphology was one of the main reasons why Schlegel and co-workers (6) originally classified their hydrogen bacterium as a corynebacterium. Both *Xanthobacter* and *Rhodopseudomonas* fix nitrogen, use molecular hydrogen as an electron donor, possess hydrogenase and catalase, produce carotenoid pigments, have the same range of G+C content (between 65 and 70 mol%), use many organic acids as substrate, assimilate few or no carbohydrates, and possess the Entner-Doudoroff pathway. The position of *R. gelatinosa* is exceptional. We tested four strains of this species. On the rRNA similarity map of *B. indica* (Fig. 3), they are all located at a $T_{m(e)}$ of 57 to 59°C, quite out of the range of the other *Rhodopseudomonas* species. From hybridizations with other reference rRNA's, we know that these strains are not related to the *Enterobacteriaceae*, the *Vibrionaceae*, the *Rhizobiaceae*, *Chromobacterium*, *Janthinobacterium*, the acetic acid bacteria, and many other taxa (M. Gillis P. De Vos, J. De Smedt, and J. De Ley, manuscript in preparation). Hybridizations with ¹⁴C-labeled rRNA from *Pseudomonas acidovorans* ATCC 15668 have shown that the rRNA cistrons of the *R. gelatinosa* strains have closer similarity ($T_{m(e)}$, from 74.5 to 75.5°C) to the rRNA cistrons of *Pseudomonas acidovorans* (De Vos and De Ley, manuscript in preparation). Obviously, *R. gelatinosa* is generically misnamed and belongs in a different rRNA superfamily. Its exceptional taxonomic position is in excellent agreement with phenotypic information (N. Pfennig, personal communication)

and with amino acid sequence data (2). Cytochrome c_2 of authentic rhodopseudomonads is most similar to cytochrome c_{550} of *Paracoccus denitrificans*. *R. gelatinosa* cytochrome c_2 , however, more closely resembles cytochrome c_{551} of *Pseudomonas*. Our data suggest that *R. gelatinosa* should be moved to another genus. The probable kinship between *Paracoccus denitrificans* and authentic rhodopseudomonads is confirmed by DNA-rRNA hybridizations; they are located in the same region on the rRNA similarity map and belong in our fourth rRNA superfamily (Fig. 3). Our results show that there are unexpected distinct generic similarities between authentic *Rhodopseudomonas* strains and our fourth rRNA superfamily (*Agrobacterium*, *Rhizobium*, *Acetobacter*, *Gluconobacter*, *Zymomonas*, *Beijerinckia*, *Azospirillum*, etc.). This tends to upset the widespread view that phototrophic bacteria constitute a separate phylogenetic branch, entirely different from the ordinary chemoorganotrophic bacteria and most closely related to the primitive bacteria from the Precambrian period.

"*Pseudomonas*" *azotocolligans* was described by Anderson (3) as a nitrogen-fixing, polarly flagellated organism. However, Hill and Postgate (30) found no nitrogen fixation for "*Pseudomonas*" *azotocolligans* NCIB 9391, and De Ley (15) showed that the same strain was peritrichous. On the basis of flagellation, this organism was misidentified. The results of the DNA-rRNA hybridizations agree perfectly with the latter conclusion.

"*Pseudomonas*" *diminuta* is phenotypically (4) and on the basis of DNA homology (37) and rRNA homology (38) quite different from the other *Pseudomonas* groups. This organism does not utilize carbohydrates, only some alcohols and organic acids. Physiologically it most closely resembles *Gluconobacter* (4). The rRNA cistrons show remote similarity to those of *Acetobacter* and *Gluconobacter* (Gillis and De Ley manuscript in preparation). Our results agree with these findings.

Previously, we have shown that "*Agrobacterium*" *aggregatum* does not belong in *Agrobacterium* (23). This organism is located on the rRNA similarity map of *B. indica* NCIB 8712 at a $T_{m(e)}$ of 72°C. Two other misidentified organisms ("*Agrobacterium*" *gelatinovorum* and "*Agrobacterium*" *luteum*) are located in the same rRNA superfamily.

Azospirillum belongs in the same rRNA superfamily as *Beijerinckia* (Fig. 3). The rRNA cistrons of *Azospirillum lipoferum* and *Azospirillum brasilense* are very similar (Table 1). The interspecies DNA homology in this genus was 30

to 50% (44). A similar situation was described previously for *Agrobacterium* cluster I (23); organisms with 45% or more DNA homology have almost identical rRNA cistrons. The closest relatives of *Azospirillum* now known are *S. itersonii* subsp. *vulgatum* and *S. polymorphum*. Although these organisms belong in one rRNA group, the inclusion of the nitrogen-fixing azospirilla in a separate genus (44) seems justified; with respect to their rRNA cistrons, they differ as much from the authentic spirilla as *Azotobacter* differs from *Alteromonas communis* and *Alteromonas vaga*. The *Azospirillum* strains and some *Spirillum* strains investigated constitute a separate branch in our fourth rRNA superfamily.

Derxia is the fourth genus included in the family *Azotobacteriaceae* in *Bergey's Manual*, 8th ed. (13). Its rRNA cistrons are quite different from those of *Azotobacter*, *Azomonas*, and *Beijerinckia*. From the hybridizations between the DNAs from three *D. gummosa* strains and the ¹⁴C-labeled rRNA's from a great variety of other organisms (Tables 1 and 2), it is quite obvious that the *Derxia* rRNA cistrons most closely resemble those of *Pseudomonas acidovorans*, *Pseudomonas solanacearum*, *Chromobacterium violaceum*, *Janthinobacterium lividum*, and *Alcaligenes faecalis*. These taxa, together with a few others, constitute our third rRNA superfamily, which consists of gram-negative rods which are usually 0.5 to 1 by 1 to 4 μ m, do not have resting stages, have 57 to 72 mol% G+C, are chemo-organotrophic, exhibit respiratory metabolism, and occur in soil and water (19).

Biological fixation of molecular nitrogen can be performed by a great variety of procaryotic microorganisms (39, 42). Most of the aerobic, free-living, nitrogen-fixing bacteria have been assembled in the family *Azotobacteriaceae*. In the 6th (10) and 7th (11) editions of *Bergey's Manual*, this family contained only one genus, *Azotobacter*, and three species, *Azotobacter chroococcum*, *Azotobacter agilis*, and *Azotobacter indicus*. In the 8th edition of *Bergey's Manual* (13), there are four genera in the family *Azotobacteriaceae*: *Azotobacter*, *Azomonas*, *Beijerinckia*, *Derxia*. There appear to be two main reasons why these genera are maintained in one family: (i) they all fix nitrogen, and (ii) three of the four genera (*Azotobacter*, *Azomonas*, and *Beijerinckia*) are based on species which had been placed in the genus *Azotobacter* (11). No other specific fundamental features except Gram negativity, strict aerobiosis, and chemo-organotrophy support the classification of these four genera into one family. These gen-

era are phenotypically very heterogeneous in cell size and shape, cyst formation, type of flagellation, lipid inclusion, manner of growth, G+C composition of DNA, etc. (19, 27, 29, 30, 45). Previous experience in our laboratory has shown that organisms which belong to a well-established, phenotypic family, such as the *Rhizobiaceae* (23), the *Enterobacteriaceae*, or the *Vibrionaceae* (De Ley, Tytgat, and De Smedt, manuscript in preparation), form DNA-rRNA hybrids with a $T_{m(e)}$ of at least 68°C. *Derxia*, *Beijerinckia*, *Xanthobacter*, and *Azospirillum* are all at a $T_{m(e)}$ below 66°C versus *Azotobacter*. They can thus not belong in the same family as *Azotobacter*. The free-living, nitrogen-fixing bacteria occur in three rRNA superfamilies. These results induce us to conclude either that the present family *Azotobacteriaceae* does not exist as a biological unit or that it should be limited to the genera *Azotobacter* and *Azomonas*.

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REPRINT REQUESTS

Address reprint requests to: Prof. J. De Ley, Laboratory of Microbiology, Rijksuniversiteit, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium.

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