Intra- and Intergeneric Similarities of *Agrobacterium* Ribosomal Ribonucleic Acid Cistrons

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We prepared hybrids between ^{14}C -labeled ribosomal ribonucleic acid (rRNA) from either *Agrobacterium tumefaciens* ICPB TT111 or *A. rhizogenes* ICPB TR7, and deoxyribonucleic acid (DNA) from a great variety of reference gram-negative and gram-positive bacteria. Each hybrid was described by (i) its $T_{m(e)}$, the temperature at which 50% of the hybrid was denatured, and (ii) percentage of rRNA binding, i.e., micrograms of ^{14}C -labeled rRNA duplexed per 100 μg of filter-fixed DNA. Each taxon occupied a definite area on the rRNA similarity map. The size and shape of this area depended on the phenotypic and genetic heterogeneity of the taxon. There appeared to be a correlation between $T_{m(e)}$ of the heterologous hybrids and the overall phenotypic similarities of the organisms and taxa involved. $T_{m(e)}$ values above 65°C were taxonomically most meaningful. DNA:rRNA hybridizations condensed all strains from a genus in one narrow cluster; the method had little resolution to distinguish species within a genus, but it seemed to be a very useful approach to detect remote relationships at the inter- and suprageneric level, for taxonomic and identification purposes. The hybrid parameters of *Azotomonas fluorescens*, *Mycoplana bullata*, *Mycoplana dimorpha*, *Phyllobacterium*, two misnamed "*Chromobacterium lividum*" strains from leaf-nodulating plants, two misnamed agrobacteria from the Baltic Sea, and a few misnamed "*Achromobacter*" strains were all in the vicinity of *Agrobacterium* and *Rhizobium*. We suggest that all of these organisms are remote relatives and belong in the family of the *Rhizobiaceae*. *Azotomonas insolita* NCIB 9749 is misnamed; it is an *Agrobacterium*. Several organisms which had been misnamed *Agrobacterium* formed DNA:rRNA hybrids with properties outside the *Agrobacterium* area.

The nature and degree of the phylogenetic relationships between most bacterial genera are not known, except for a limited number of cases, where such relationships can be deduced indirectly from comparative biochemistry (13), numerical analysis of phenotypic data (65), and deoxyribonucleic acid (DNA):DNA hybridizations (14, 21). Most bacterial genera, however, are evolutionarily too far removed from each other to form stable DNA:DNA hybrids; the last technique is in general useful either within a genus such as *Agrobacterium* (16, 17), or between genera which did not diverge too much, such as in the *Enterobacteriaceae* (7).

The detection of more remote relationships between bacterial taxa will require other techniques. One of them is the comparison of amino acid sequences of small proteins. It is well established that the amino acid sequences of mitochondrial *c* cytochromes from animals correlate well with the overall phylogenetic relationships of the organisms, clarified by other methods (12, 49). The other way around, amino acid

sequencing of bacterial cytochromes and other small proteins might reveal the phylogenetic relationships between bacterial genera (12). This area is in full expansion (3). However, it may take a very long time and much effort to solve this single aspect of bacterial relationships.

It seemed to us that a faster way than determining amino acid sequences in homologous gene products consisted in comparing base sequence similarities between homologous genes. The advantage of the latter approach was that the actual sequences need not to be known. The obvious choice was to compare ribosomal ribonucleic acid (rRNA) cistrons from several genera by DNA:rRNA hybridizations. rRNA cistrons are conserved: their base sequences have changed less than those of the bulk of the genes constituting the bacterial genome (26, 27, 53, 71). Various degrees of rRNA sequence similarities have been detected between phenotypically quite different bacteria (39, 53, 54, 56, 57), but extensive taxonomic improvements

have not been achieved. rRNA homology, determined by preventing homologous DNA:rRNA duplexing in the presence of excess heterologous competing rRNA, is useful to subdivide large and heterogeneous genera, such as *Pseudomonas* (58) and *Clostridium* (40).

We shall examine whether bacterial rRNA similarities are valuable parameters to reveal taxonomic relatedness at the generic and suprageneric levels. In the present paper we shall compare the rRNA cistrons of *Agrobacterium* within the genus and with those of a number of other bacterial genera. Several conclusions were confirmed by DNA:DNA hybridizations and by protein electropherograms.

MATERIALS AND METHODS

Bacterial strains and growth media. The strains used are listed in Table 1 (see also Table 6). The bacteriological purity was checked by plating, and by examination of living and gram-stained cells. For mass cultures, cells were grown in Roux flasks on media solidified with 2.5% agar for 2 to 3 days at 28°C. A few slow-growing strains were grown for at most 4 to 6 days. The compositions of all growth media used are summarized in Table 2. After growth the cells were harvested, washed, and lyophilized.

We received three *Phyllobacterium* strains from D. Knösel, *P. rubiacearum* strain LMG1 and *P. myrsinacearum* LMG2 and LMG3. Upon plating we detected two colony types in each strain, labeled t_1 and t_2 . Strain LMG1 t_2 (sequence number 11 [see Table 1]; henceforward, these numbers will be indicated as, for example, "number 11" following the strain, was identified in this laboratory as a contaminating *Agrobacterium*. The five remaining strains (numbers 50 to 54) were included as *Phyllobacterium*.

Preparation of ^{14}C -labeled rRNA. The organisms were inoculated in 100 ml of liquid medium A (Table 2), in a broad-bottomed Erlenmeyer flask with a Klett tube as side arm. Shaking provided good aeration. Growth was followed turbidimetrically in a Klett colorimeter at 660 nm. A 100- μCi amount of [2- ^{14}C]uracil (The Radiochemical Centre, Amersham, Buckinghamshire, England) in 5 ml of 0.01 M phosphate buffer (pH 7.0) was sterilized separately through a membrane filter and added at the start of the log phase. The cells were harvested near the end of the exponential phase and washed. ^{14}C -labeled rRNA was prepared and purified as described by De Ley and De Smedt (20). The 23S (see below) and 16S fractions were collected separately and dialyzed at 4°C three times against 2 liters of $2\times$ SSC buffer. The fractions were preserved at -12°C. The specific activities of *Agrobacterium tumefaciens* TT111 ^{14}C -labeled rRNA and of *Agrobacterium rhizogenes* TR7 ^{14}C -labeled rRNA were 3,000 cpm/ μg and 2,400 cpm/ μg , respectively. These preparations contained less than 0.2% DNA and 1% protein.

Preparation of DNA. High-molecular-weight native DNA was prepared by a combination of the methods of Marmur (50) and Kirby (43, 44), as de-

scribed by De Ley et al. (19). Several gram-positive and coryneform organisms lysed readily in the solvent described by Crombach (11): 0.033 M tris-(hydroxymethyl)aminomethane-0.001 M ethylenediaminetetraacetic acid (pH 8), containing 5 mg of lysozyme per g of wet cells. The molecular weight of the DNA fragments was 5×10^6 to 10×10^6 .

Fixation of single-stranded high-molecular-weight DNA on membrane filters. We followed the fixation procedure as described by De Ley and Tytgat (24). We used Sartorius SM 11309 membrane filters. The charged filters were preserved at 4°C in vacuo (20).

Saturation hybridization between ^{14}C -labeled rRNA and filter-fixed DNA: thermal stability of the DNA:rRNA hybrids. The basic aspects of the hybridization conditions, the nature of ribonuclease and its effect on hybridization, the effect of hybridization temperature on DNA leaching, the conditions of saturation hybridization, etc., have been reported in previous papers from this department (20, 24). For DNA:rRNA hybridization and hybrid stability we followed the methods of De Ley and De Smedt (20). A 10- μg amount of ^{14}C -labeled rRNA was incubated in 1 ml of $2\times$ SSC in 20% formamide, with a membrane filter carrying about 50 μg of DNA, for 16 h at the stringent optimal hybridization temperature of 50°C. After washing, the filters were treated with RNase. The thermal stability of the hybrid was determined in 5-degree steps from 50 to 90°C in $1.5\times$ SSC in 20% formamide. The amount of labeled material released at each step was counted in a Tri-Carb 3310 liquid scintillation spectrometer (Packard Instrument Co.) at 2°C for 50 min. T_{me} is the temperature at which 50% of the hybrid was eluted. The total amount of ^{14}C -labeled rRNA bound, after RNase treatment, expressed in micrograms of rRNA per 100 μg of DNA retained on the filter, was called the "percentage of rRNA binding."

Chemical determination of DNA on the filter. In each series of hybridizations, a vial was included with buffer and a DNA filter, but without ^{14}C -labeled rRNA. After the simulated hybridization step, the remaining fixed DNA was released from the filter by the method of Meys and Schilperoort (51) and determined by the method of Burton (10).

Preparation of ^{14}C -labeled DNA. Labeled DNA was prepared as described before (35). The specific activities of ^{14}C -labeled DNA from *A. tumefaciens* ICPB TT111 and B6 were 4,670 and 3,965 cpm/ μg of DNA, respectively. Both were sheared in a French pressure cell to fragments of about 4×10^6 molecular weight and were heat-denatured before use.

DNA:DNA hybridizations: thermal stability of the DNA:DNA duplexes. We followed the method of De Ley and Tytgat (24), slightly modified, with 10 μg of single-stranded, sheared, ^{14}C -labeled DNA in solution, and a membrane filter with about 22 μg of single-stranded, high-molecular-weight, unlabeled DNA in 0.8 ml of $2\times$ SSC buffer in 30% dimethyl sulfoxide (Me_2SO). Incubation was for 16 h at the stringent temperature of 59°C. After washing each filter in $2\times$ SSC-30% Me_2SO , the thermal stability of each duplex was determined in the same Me_2SO -

TABLE 1. List of organisms used, their strain number, growth medium, DNA base composition, and properties of the DNA:rRNA hybrids with two *Agrobacterium* ¹⁴C-labeled rRNA references

No. in Fig. 3 or 4	Organism used for DNA prepn	Origin and strain no.	Growth medium ^a	Mol % G+C ^b	Hybridization with rRNA from:			
					<i>Agrobacterium tumefaciens</i> TT111		<i>Agrobacterium rhizogenes</i> TR7	
					<i>T_m(°C)</i>	% binding	<i>T_m(°C)</i>	% binding
1	<i>Agrobacterium tumefaciens</i>	ICPB TT111	A	60.6	81	0.19	77	0.20
2	<i>Agrobacterium tumefaciens</i>	ATCC 4452	A	60.9	80	0.19		
3	<i>Agrobacterium tumefaciens</i>	ATCC 11156	A	60.8	81	0.14		
4	<i>Agrobacterium tumefaciens</i>	Beardsly H5	A	59.7	79	0.18	76	0.18
5	<i>Agrobacterium tumefaciens</i>	ICPB TT7	A	59.6	81	0.24		
6	<i>Agrobacterium tumefaciens</i>	C-58	A	59.9	80.5	0.22		
7	<i>Agrobacterium tumefaciens</i>	T37	A	60.2	79.5	0.13	77	0.16
8	<i>Agrobacterium tumefaciens</i>	3/1	A	61.6	80	0.24	77.5	0.22
9	<i>Agrobacterium tumefaciens</i>	NCPPB 925	A	60.1	80	0.20	77	0.20
10	<i>Agrobacterium tumefaciens</i>	CIP B6	A	60.2			77	0.16
11	<i>Agrobacterium</i> sp.	LMG1 t2	B	60.3	80.5	0.13		
12	<i>Agrobacterium</i> sp.	0363	A	60.3	79.5	0.21	76.5	0.18
13	<i>Agrobacterium</i> sp.	4.1	C	62.5	80.5	0.14	77	0.15
14	<i>Agrobacterium radiobacter</i>	M2/1	A	61.0	80	0.18	77	0.18
15	<i>Agrobacterium radiobacter</i>	ATCC 13332	A	60.1			77	0.22
16	<i>Agrobacterium tumefaciens</i>	NCPPB 794	A	61.3	77	0.15	80.5	0.17
17	<i>Agrobacterium rhizogenes</i>	ICPB TR7	A	61.4	78	0.12	81	0.15
18	<i>Agrobacterium rhizogenes</i>	ICPB TR101	A	60.7	77	0.09	81	0.16
19	<i>Agrobacterium rhizogenes</i>	ATCC 15834	A	60.5			81	0.12
20	<i>Agrobacterium rubi</i>	ICPB TR2	A	58.8	80.5	0.20	76	0.15
21	<i>Agrobacterium tumefaciens</i>	EU6	A	58.8	77.5	0.19		
22	<i>Agrobacterium tumefaciens</i>	NCPPB 1771	A	58.0	76	0.23	76	0.22
23	<i>Agrobacterium tumefaciens</i>	NCPPB 1650	A	57.8	78	0.18	76	0.18
24	<i>Agrobacterium tumefaciens</i>	NCPPB 930	A	58.9	78	0.16	76	0.18
25	<i>Agrobacterium</i> sp.	175/1	A	57.4	78	0.11		
26	<i>Agrobacterium</i> sp.	177/14	A	57.6	78	0.12		
27	" <i>Agrobacterium</i> " <i>ferrugineum</i>	Ahrens A43	T	64.8	73.5	0.06		
28	" <i>Agrobacterium</i> " <i>kielense</i>	Ahrens B9	T	50.6	72	0.08		
29	" <i>Agrobacterium</i> " <i>luteum</i>	Ahrens B14	T	52.5	67	0.06		
30	" <i>Agrobacterium</i> " <i>gelatinovorum</i>	Ahrens B6	U	57.6	67	0.07		
31	" <i>Agrobacterium</i> " <i>aggregatum</i>	Ahrens B1	T	58.7	67.5	0.06		
32	" <i>Agrobacterium</i> " <i>agile</i>	Ahrens A82	T	57.1	63.5	0.09		
33	" <i>Agrobacterium</i> " sp.	Sundman 08 = 113	A	62.0	61	0.07		
34	" <i>Agrobacterium</i> " sp.	Sundman M14 = 112	A	42.9	57	0.11		
35	" <i>Agrobacterium</i> " <i>azotophilum</i>	(Ulloa)	A	63.5	57	0.06		
36	<i>Rhizobium leguminosarum</i>	USDA 316C10A	C	61.4	76	0.08	80	0.15
37	<i>Rhizobium leguminosarum</i>	11.1	C	62.1	77	0.12	79.5	0.12
38	<i>Rhizobium leguminosarum</i>	5.0	C	61.1	76	0.11	79	0.14
39	<i>Rhizobium leguminosarum</i>	USDA 3F3C1	D	62.5	70.5	0.06	72	0.07
40	<i>Rhizobium leguminosarum</i>	USDA 3F6g2	C	61.3	72.5	0.06	74	0.06
41	<i>Rhizobium meliloti</i>	USDA 3D0a30	D	62.3	77	0.10	79	0.12
42	<i>Rhizobium japonicum</i>	3.1	I	64.0	70	0.06	70	0.06
43	<i>Rhizobium japonicum</i>	USDA 316n10	I	63.3	67	0.03	70	0.04
44	<i>Rhizobium japonicum</i>	USDA 3I1b59	I	64.0	65	0.04	68	0.05
46	<i>Azotomonas fluorescens</i>	NCIB 9884	B	64.8	76	0.10		
47	<i>Azotomonas insolita</i>	NCIB 9749	B	58.9	80	0.13		
48	<i>Mycoplana bullata</i>	NCIB 9440	B	62.3	77	0.10	77	0.08
49	<i>Mycoplana dimorpha</i>	NCIB 9439	B	63.8	75	0.07	76.5	0.11
50	<i>Phyllobacterium rubiacearum</i>	LMG1 t1	B	61.3	72	0.07		
51	<i>Phyllobacterium myrsinacearum</i>	LMG2 t1	B	60.3	73	0.08		
52	<i>Phyllobacterium myrsinacearum</i>	LMG2 t2	B	59.8	73.5	0.08		
53	<i>Phyllobacterium myrsinacearum</i>	LMG3 t1	B	60.4	73	0.08		
54	<i>Phyllobacterium myrsinacearum</i>	LMG3 t2	B	59.6	72.5	0.08		
55	<i>Acetobacter aceti</i> subsp. <i>aceti</i>	Ch 31	N	59.6	66.5	0.09		
56	<i>Gluconobacter oxydans</i> subsp. <i>melanogenes</i>	NCIB 8086	N	60.6	67	0.09		

TABLE 1—Continued

No. in Fig. 3 or 4	Organism used for DNA prep	Origin and strain no.	Growth medium ^a	Mol % G+C ^b	Hybridization with rRNA from:			
					<i>Agrobacterium</i> <i>tumefaciens</i> TT111		<i>Agrobacterium</i> <i>rhizogenes</i> TR7	
					<i>T_m(e)</i>	% bind- ing	<i>T_m(e)</i>	% bind- ing
57	<i>Zymomonas mobilis</i> subsp. <i>mobilis</i>	Z1	Y	48.8	68	0.16		
58	<i>Beijerinckia fluminensis</i>		V	56.2	67	0.12		
59	<i>Beijerinckia indica</i>		V	54.7	67.5	0.09		
60	" <i>Chromobacterium</i> " <i>lividum</i>	NCTC 10590	X	61.1	66.5	0.06		
61	" <i>Chromobacterium</i> " <i>lividum</i>	NCTC 10591	X	63.0	67.5	0.08		
62	<i>Chromobacterium lividum</i>	NCTC 9796	X	65.5	59	0.11		
63	<i>Chromobacterium lividum</i>	Sneath DA	X	66.1	58	0.10	57.5	0.17
64	<i>Chromobacterium violaceum</i>	NCTC 9757	X	67.2	60	0.15		
65	" <i>Achromobacter</i> " <i>hartlebii</i>	NCIB 8129	B	62.8	77.5	0.09		
66	" <i>Achromobacter</i> " sp.	AB 1196	A	59.2	73	0.12	73	0.17
67	" <i>Alcaligenes</i> " sp.	AB 940	A	58.0	73	0.12		
68	" <i>Achromobacter</i> " sp.	AB 1293	A	58.8	72.5	0.10		
69	" <i>Achromobacter</i> " sp.	Ruiter 1	B	53.0	63.5	0.11		
70	" <i>Achromobacter</i> " sp.	Ruiter 5	B	53.7	62	0.11		
71	<i>Achromobacter denitrificans</i>	M 250	B	67.7	60	0.04	58.5	0.09
72	<i>Achromobacter xylosoxydans</i>	KM 583	B	69.5	59	0.05	60	0.06
73	<i>Alcaligenes faecalis</i>	AB 1286	A	57.2	56.5	0.04	58	0.12
74	<i>Alcaligenes odorans</i>	Gilardi 117	A	57.9	57	0.06	58	0.08
75	" <i>Alcaligenes</i> " <i>paradoxus</i>	ATCC 17712	B	67.9	61	0.05		
76	<i>Escherichia coli</i>	B	R	52.2	62.5	0.11		
77	<i>Enterobacter aerogenes</i>	NCTC 10006	J	53.8	63	0.11		
78	<i>Enterobacter agglomerans</i> ("Agrobacterium gypsophi- lae")	IPO 445	B	60.6	62	0.12		
79	("Agrobacterium gypsophi- lae")	ATCC 13329	B	56.2	63.5	0.12		
80	("Agrobacterium gypsophi- lae")	IPO 280e	B	55.7	63.5	0.12		
81	<i>Erwinia amylovora</i>	NCPFB 683	B	54.0	61.5	0.12	60.5	0.17
82	<i>Erwinia lathyri</i>	PA	B	56.5			60	0.17
83	<i>Klebsiella pneumoniae</i>	N4B	J	58.6	62	0.10	62	0.15
84	<i>Salmonella typhimurium</i>	1	B	55.2	62	0.11		
85	<i>Salmonella pullorum</i>	3	W	54.0			61	0.15
86	<i>Aeromonas formicans</i>	NCIB 9232	F	64.8	60	0.13		
87	<i>Aeromonas salmonicida</i>	NCMB 833	G	57.0	59	0.13		
88	<i>Pseudomonas diminuta</i>	CCEB 513	B	67.3	65	0.06		
89	<i>Pseudomonas aeruginosa</i>	NCTC 7244	B	66.8	62.5	0.06		
90	<i>Pseudomonas aureofaciens</i>	CCEB 518	B	62.8	59.5	0.08		
91	<i>Pseudomonas cichorii</i>	NCPFB 906	B	59.7	61.5	0.11	60	0.10
92	<i>Pseudomonas coronafaciens</i>	NCPFB 1328	L	59.3	62.5	0.08		
93	<i>Azotobacter paspali</i>	22B	M	63.7	59.5	0.09		
94	<i>Azotobacter vinelandii</i>	NCIB 8660	K	65.0	62	0.07		
95	<i>Azomonas macrocytogenes</i>	NCIB 8200	K	58.6	61.5	0.07		
96	<i>Azomonas agilis</i>	SS4	H	52.8	63.5	0.08		
97	<i>Bordetella bronchiseptica</i>	ATCC 10580	B	68.2	58.5	0.06		
98	<i>Bordetella bronchiseptica</i>	NCTC 8761	B	69.5	60	0.06		
99	<i>Campylobacter fetus</i> subsp. <i>jejuni</i>	M2		34.0	57	0.07		
100	<i>Arthrobacter oxydans</i>	CBRI 21010	O	62.4	56	0.07	59.5	0.12
101	<i>Arthrobacter polychromo- genes</i>	CBRI 21038	O	60.8	57	0.07	57	0.09
102	<i>Arthrobacter</i> sp.	CBRI ER39	O	65.1	55	0.09	56.5	0.11
103	<i>Arthrobacter</i> sp.	CBRI ER56	O	63.2	55.5	0.07	56	0.10
104	<i>Arthrobacter globiformis</i>	CBRI 21033	O	66.0	59	0.09	55	0.14
105	<i>Arthrobacter aurescens</i>	CBRI 21002	O	59.5	57	0.11	55	0.18
106	<i>Arthrobacter oxydans</i>	CBRI 21011	O	65.9	55.5	0.09	57	0.14
107	<i>Arthrobacter histidinolovor- ans</i>	CBRI 21040	O	61.3	57	0.10	55.5	0.12
108	<i>Arthrobacter</i> sp.	CBRI ER2	O	60.5	56	0.07	58	0.11
109	<i>Arthrobacter</i> sp.	CBRI ER3	O	61.0	56	0.07	56	0.10
110	<i>Arthrobacter tumescens</i>	CBRI 21016	Q	72.4	55	0.06	57	0.08
111	<i>Arthrobacter crystallopoites</i>	CBRI 21030	O	63.9	57	0.15	56.5	0.15
112	<i>Arthrobacter atrocyaneus</i>	CBRI 21001	O	70.5	57	0.08	59	0.10
113	<i>Arthrobacter flavescens</i>	CBRI 21005	P	70.3	58	0.06	58	0.06

TABLE 1—Continued

No. in Fig. 3 or 4	Organism used for DNA prep	Origin and strain no.	Growth medium ^a	Mol % G+C ^b	Hybridization with rRNA from:			
					<i>Agrobacterium</i> <i>tumefaciens</i> TT111		<i>Agrobacterium</i> <i>rhizogenes</i> TR7	
					<i>T_m(e)</i>	% bind- ing	<i>T_m(e)</i>	% bind- ing
114	" <i>Agrobacterium</i> " <i>pseudotsu- gae</i>	ATCC 13330	A		57	0.10		
115	" <i>Agrobacterium</i> " <i>pseudotsu- gae</i>	NCPPB 180	A	67.7	56.5	0.12		
116	<i>Corynebacterium glutamicum</i>	MPS 10	B	54.5			57	0.15
117	<i>Corynebacterium insidiosum</i>	Joubert A	B		55	0.04		
118	<i>Corynebacterium fascians</i>	D 188	B		54.5	0.05		
119	<i>Microbacterium flavum</i>	NCIB 8707	B	58.2	59	0.08		
120	<i>Microbacterium lacticum</i>	NCIB 8540	S	62.9	57	0.02		
121	<i>Nocardia asteroides</i>	IMET 7020	B		58.5	0.04		
122	<i>Nocardia turbata</i>	n 32	B		58	0.08		
123	<i>Mycobacterium rhodochrous</i>	01	B		58	0.05		
124	<i>Bacillus megaterium</i>	899 thy ⁻	B	39.9	60	0.19		
125	<i>Bacillus pumilus</i>	B 12	B	42.7	58	0.10		
126	<i>Bacillus subtilis</i>	BQ 2	B	47.7	59	0.16		
127	HeLa			40.0		0		

^a For composition of the growth media, see Table 2.

^b The moles percent guanine plus cytosine (G+C) values were determined in our laboratory on previous occasions by thermal denaturation.

containing solvent in 3-degree temperature steps from 62 to 86°C. Radioactivity released at each temperature step was counted as above, but in Bray solution (6).

RESULTS

16S and 23S rRNA fractions. Although 23S rRNA is frequently nicked (62), it can readily be prepared intact from many bacteria. *Agrobacterium* represents an extreme case: almost no 23S RNA can be prepared from it (20, 32, 47, 48), because it contains at least two nicks (33, 62). When ¹⁴C-labeled rRNA from our reference strains *A. tumefaciens* TT111, *A. rhizogenes* TR7, and *Escherichia coli* B were separated on a sucrose gradient, and the distributions were aligned on the 16S peaks, it appeared that the agrobacterial 23S peaks were lighter and much smaller than the 23S *E. coli* peak (Fig. 1). Even when rRNA was prepared in the presence of either bentonite (52), or 2% sodium lauryl sulfate (55), the yield of the 23S fraction remained low. In view of Schuch's and Loening's (62) results, our agrobacterial 23S peaks consisted most likely of RNA with a molecular weight of 0.95×10^6 , and the 16S peaks were probably heavily contaminated with fragmented 23S. For the sake of simplicity we shall continue to use both symbols. There is no evidence that the 23S fraction would be contaminated by 16S.

Similarity between 16S and 23S rRNA and the number of rRNA cistrons in *Agrobacter-*

ium. We saturated filter-fixed DNA from both *Agrobacterium* reference strains with 16S, 23S, or the mixed preparation of homologous ¹⁴C-labeled rRNA (for method, see 20). The percentage of rRNA binding in the case of *A. tumefaciens* was 0.189 and in the case of *A. rhizogenes* it was 0.154 and 0.150 (Table 3). These results suggested that the base sequences in 16S and 23S rRNA cistrons in each strain were very similar.

Table 3 shows that the *A. tumefaciens* TT111 DNA genome probably contains a total length of rRNA cistrons equivalent to 12 16S units. Saturation hybridization indicates an equivalent of 10 16S units in *A. rhizogenes*. Cross-hybridization (Table 1, Fig. 3 and 4) confirmed this conclusion. When ¹⁴C-labeled rRNA from either *A. tumefaciens* TT111 or *A. rhizogenes* TR7 were hybridized to DNA from strains of both cluster 1 and cluster 2 (16, 17), the percentage of rRNA binding was on the average 25% smaller with the latter, confirming that bacteria from the *rhizogenes* cluster 2 have fewer rRNA cistrons per genome.

Stability of hybrids and 16S and 23S rRNA fractions. Table 4 compares the thermal stability of a number of rDNA hybrids with either 16S or 23S ¹⁴C-labeled rRNA from *A. tumefaciens* TT111. From the 11 pairs of determinations, it followed that the stability of both types of hybrids usually differs by at most 1°C. When the percentage of rRNA binding drops to 0.05 to 0.06 and below, there may be 2 to 3°C differ-

TABLE 2. Composition of growth media (in percentage weight/volume except when stated otherwise)

[illegible]

^a Except when stated otherwise.

^b Medium symbol.

ence, largely because of experimental errors. The percentage of rRNA binding was also very similar with either 16S or 23S rRNA; the difference was usually at most 0.02%.

Comparison of the rRNA hybrids. Ribosomal RNA similarity was expressed by two parameters, $T_{m(e)}$ and percent rRNA binding. The latter value depended not only on the actual rRNA homology, but also on the size of the genome, its state of replication, and the number of rRNA cistrons per genome; it represented a certain amount of rRNA bound, typical for each taxon, but it can by no means be called rRNA homology. We determined a great variety of melting curves, combinations of high and

low $T_{m(e)}$ values, high and low percent RNA binding values, and values in between. A few examples are given in Fig. 2. All parameters are compiled in Table 1. The correlations between the data were better revealed by plotting, for each labeled reference rRNA, $T_{m(e)}$ versus percent rRNA binding. The resulting graphs (Fig. 3 and 4) were called here rRNA similarity maps. A few reciprocal hybridizations between DNA from *Agrobacterium* and ^{14}C -labeled rRNA from various other genera are shown in Table 5.

Amount and stability of DNA:DNA hybrids.

A number of DNA:rRNA hybridizations suggested definite relationships between strains and taxa, which were hitherto unknown (see Discussion). We examined their overall genome similarity with *Agrobacterium* by DNA:DNA hybridizations. We included a large number of control strains, several of them phytopathogens (Table 6). When the melting curves of all of these duplexes were examined, they could be separated arbitrarily into five types (Fig. 5). First there were the homologous DNA:DNA duplexes (for example, TT111 $\times$ ^{14}C -labeled TT111 or B6 $\times$ ^{14}C -labeled B6; type I) and the heterologous duplexes between representatives of the genetic groups within cluster 1 (type II); they were included as controls. Earlier we reported extensively on the nature of the DNA:DNA duplexes within *Agrobacterium* (25). Type III melting curves were from strains which displayed a distinct genome similarity with *Agrobacterium*, such as several rhizobia, *Mycoplana*, "*Achromobacter*" *hartlebii*, and *Azotomonas fluorescens* (see Table 6). Type IV melting curves were from strains which displayed a weak to very weak but still distinct genome similarity with *Agrobacterium*, such as some remote rhizobia, *Phyllobacterium*, the strains AB 940, AB 1196, and AB 1293, etc. All of the other strains formed no DNA:DNA complexes with *Agrobacterium* (type V).

FIG. 1. ^{14}C -labeled rRNA distributions in a linear 15 to 30% sucrose gradient.

TABLE 3. Estimation of the number of rRNA cistrons in *Agrobacterium*^a

^{14}C -labeled rRNA from:	Mol wt of genome DNA	^{14}C -labeled rRNA prepn	% rRNA binding	Total mol wt of rRNA bound per DNA genome	Total no. of rRNA cistrons per DNA genome expressed as 16S units
<i>A. tumefaciens</i> ICPB TT111	3.4×10^9	16S	0.189	6.43×10^6	12
		23S	0.189	6.43×10^6	
		16S + 23S	0.189	6.43×10^6	
<i>A. rhizogenes</i> ICPB TR7	3.6×10^9	16S	0.154	5.54×10^6	10
		23S	0.150	5.4×10^6	

^a The molecular weight of the genome DNA was determined with the initial renaturation rate method (28).

TABLE 4. Comparison of the stability of hybrids with either 16S or 23S ^{14}C -labeled rRNA from *Agrobacterium tumefaciens* ICPB TT111^a

DNA from:	16S		23S	
	$T_{m(e)}$	%	$T_{m(e)}$	%
<i>Agrobacterium tumefaciens</i> ICPB TT111	81	0.19	81	0.19
<i>Agrobacterium</i> sp. 4.1	81	0.14	81	0.13
<i>Agrobacterium rhizogenes</i> ICPB TR7	78	0.12	77.5	0.10
<i>Rhizobium meliloti</i> USDA 3D0a30	77	0.10	78	0.08
<i>Rhizobium leguminosarum</i> USDA 316C10A	76	0.08	77	0.09
<i>Rhizobium leguminosarum</i> 11.1	77	0.12	77.5	0.10
<i>Rhizobium leguminosarum</i> 5.0	76	0.11	77	0.09
<i>Rhizobium leguminosarum</i> USDA 3F3C1	71	0.06	74	0.06
<i>Rhizobium leguminosarum</i> USDA 3F6g2	72.5	0.06	75	0.05
<i>Arthrobacter polychromogenes</i> CBRI 21038	57	0.07	54	0.07
<i>Arthrobacter oxydans</i> CBRI 21011	55.5	0.09	53.5	0.09

^a The results are expressed in percent rRNA binding and as $T_{m(e)}$ of the hybrids in $^{\circ}\text{C}$.

FIG. 2. Examples of melting curves of hybrids between 16S ^{14}C -labeled rRNA from *Agrobacterium tumefaciens* TT111 and filter-fixed DNA from various bacteria. The total amount of cp50m/100 μg of DNA (counts per 50 min per 100 μg of filter-fixed DNA) is a measure of the amount of hybrid denatured. The melting point $T_{m(e)}$ is represented by the central dot on each curve.

DISCUSSION

When the results were plotted in an rRNA similarity map, each strain had a fixed position (within the limits of reproducibility, $T_{m(e)} \pm 0.5^{\circ}\text{C}$; percent rRNA binding $\pm 0.015\%$). Strains of the same species, which are phenotypically and genotypically very similar, are almost indistinguishably together on the map. Examples are the four strains of *Phyllobacterium myrsinacearum* (numbers 51 to 54), three strains of *Enterobacter agglomerans* (numbers 78, 79, and 80), and both strains of *Chromobacterium lividum* (numbers 62 and 63). Species from the same genus are close together within a limited area. In several cases, only a small number of strains were included merely to locate the position of the genus on the map, not its area shape. When more strains are used, the shape and size of the area occupied by a genus

is a measure of its heterogeneity. From previous research in this laboratory (16, 17, 25, 42) we know that *Agrobacterium* is a rather heterogeneous genus, consisting of two large genetic clusters, 1 (exemplified by *Agrobacterium radiobacter* and *A. tumefaciens* strains, numbers 1 to 15) and 2 (exemplified by the rhizogenic and atypical *A. tumefaciens* strains, numbers 16 to 19), a small *Agrobacterium rubi* cluster (numbers 20 and 21), and a few isolated strains (numbers 22, 23, and 24). These clusters hybridize at about 15% DNA homology among each other. Cluster 1 consists of seven genetic races called TT111 (numbers 1, 5, and 11), B6 (numbers 2, 3, 4, 10, 15), TT9 (numbers 6, 7, and 13), 3/1 (number 8), F/1 (number 9), 0362 (number 12), and M2/1 (number 14). These seven genetic races hybridize among each other with 45 to 50% DNA homology. Both clusters 1 and 2 occupy separate areas on our maps (Fig. 3 and 4).

FIG. 3. Similarity map of hybrids between the 16S ^{14}C -labeled rRNA fraction of *Agrobacterium tumefaciens* ICPB TT111 and DNA from a variety of bacteria. $T_{m(e)}$ and % rRNA binding are as defined in the text. To simplify the drawing each strain is represented by a sequence number (Table 1), which is not the strain number. The positions of all strains, belonging phenotypically to the same taxon (usually a genus), are surrounded by a closed line. These areas locate the taxa on the map; their shapes and dimensions are limited by the numbers of strains used; the line is not the ultimate border.

The genetically separate strains *A. tumefaciens* NCPPB 1771 (number 22), NCPPB 1650 (number 23), and NCPPB 930 (number 24) are outside the areas occupied by cluster 1 or 2. In

1973 a severe outbreak of cane gall occurred on red raspberries in Scotland, occasioned by *Agrobacterium* sp. (M. Pérombelon, personal communication). These strains (numbers 25

FIG. 4. Similarity map of hybrids between the 16S ^{14}C -labeled rRNA fraction of *Agrobacterium rhizogenes* ICPB TR7 and DNA from a variety of bacteria. For further details, see legend to Fig. 3.

and 26) are phenotypically rather separate, fitting in none of our genetic races (K. Kersters and J. De Ley, unpublished data). The rRNA hybridization data confirmed that they are agrobacteria.

The rhizobia consist of two main subgroups: the fast-growing peritrichously flagellated organisms and the slow-growing subpolarly flagellated group (9, 23, 29). Representative strains of each subgroup were included. The peritrichous subgroup (strains 36 to 41) is probably very heterogeneous. DNA:DNA hybridizations below the optimal renaturation temperature allow more mismatches and are thus able to detect more remote relationships. In this fashion Heberlein et al. (35) found that the fast-growing rhizobia have definite genetic similarities with cluster 2 of *Agrobacterium* and that

the slow-growing rhizobia are separate. Our present results point definitely in the same direction. The similarity area of the rRNA cistrons of peritrichously flagellated *Rhizobium* strains borders on and even overlaps with the area of cluster 2 (Fig. 3 and 4). The rRNA cistrons of the slow-growing rhizobia (numbers 42, 43, and 44) are much less similar.

Is there a correlation between the rRNA similarities and other features of the genera examined? We were unable to correlate the percentage of rRNA binding with phenotypic features of the genera examined. For example, *Agrobacterium* has percent rRNA binding values in the same range as the *Enterobacteriaceae*, *Chromobacterium*, *Bacillus*, and some *Arthrobacter* species. There are but limited phenotypic similarities between these taxa. However,

TABLE 5. Hybridizations between DNA from a few *Agrobacterium* strains and ¹⁴C-labeled rRNA from various reference strains^a

DNA from:	¹⁴ C-labeled rRNA from:															
	<i>Escherichia coli</i> B		<i>Pseudomonas fluorescens</i> ATCC 13525		<i>Pseudomonas acidovorans</i> ATCC 15668		<i>Chromobacterium lividum</i> NCTC 9796		<i>Chromobacterium violaceum</i> NCTC 9757		<i>Gluconobacter oxydans</i> NCIB 9013		<i>Xanthomonas campestris</i> NCPPB 528		<i>Arthrobacter oxydans</i> CBRI 21010	
	<i>T</i> _{m(e)} ^b	% ^c	<i>T</i> _{m(e)}	%	<i>T</i> _{m(e)}	%	<i>T</i> _{m(e)}	%	<i>T</i> _{m(e)}	%	<i>T</i> _{m(e)}	%	<i>T</i> _{m(e)}	%	<i>T</i> _{m(e)}	%
<i>Agrobacterium tumefaciens</i> ICPB TT111	63.5	0.07	59	0.07	56	0.04	59.5	0.07			65	0.12			55.5	0.08
<i>Agrobacterium</i> sp. LMG1	59.5	0.05									65.5	0.11				
<i>Agrobacterium tumefaciens</i> t ₂	61	0.07	60	0.07												
<i>Agrobacterium tumefaciens</i> ATCC 11156																
<i>Agrobacterium tumefaciens</i> B2A							59.5	0.07					59.5	0.06		
<i>Agrobacterium</i> sp. 175/1											67	0.10				
<i>Agrobacterium</i> sp. 177/14											66	0.09				
<i>Agrobacterium rhizogenes</i> ICPB TR7	61	0.08	59.5	0.05			57.5	0.06							58.0	0.04
<i>Agrobacterium rhizogenes</i> ICPB TR101									59.5	0.04	66.5	0.07				

^a From J. De Ley, J. De Smedt, P. De Vos, M. Gillis, and P. Segers, unpublished data.

^b *T*_{m(e)} is expressed in °C.

^c %, Percent rRNA binding.

TABLE 6. Amount and thermal stability of stable DNA:DNA duplexes between two reference *Agrobacterium* strains and several other organisms^a

Organism	<i>A. tumefaciens</i> TT111 ¹⁴ C-labeled DNA		<i>A. tumefaciens</i> B6 ¹⁴ C-labeled DNA		Type of melting curve (see Fig. 5)	
	% ¹⁴ C-la- beled DNA bound vs. homolo- gous DNA	<i>T</i> _{m(e)} ^b	% ¹⁴ C-la- beled DNA bound vs. homolo- gous DNA	<i>T</i> _{m(e)}		
<i>Agrobacterium tumefaciens</i> ICPB TT111	(100)	75.3	58	68.4	I	II
<i>Agrobacterium tumefaciens</i> B6	51	69.5	(100)	75.4	II	I
<i>Agrobacterium tumefaciens</i> IPO 417	84	75.3			I	
<i>Agrobacterium rhizogenes</i> ICPB TR101	6	Low ^c	11	Low	IV	
<i>Agrobacterium tumefaciens</i> NCPPB 1649	6	63.2	11	Low	IV	
<i>Azotomonas insolita</i> NCIB 9749			45	68.4	II	
<i>Azotomonas fluorescens</i> NCIB 9884			7	64.9	III	
<i>Rhizobium leguminosarum</i> USDA 316C10A	11	65.0			III	
<i>Rhizobium leguminosarum</i> USDA 3F3C1	6	63			IV	
<i>Rhizobium meliloti</i> 1.5	11	64.3			III	
<i>Rhizobium meliloti</i> USDA 3D0a30	6	63.8			IV	
<i>Rhizobium japonicum</i> USDA 3I1b59	3	Low			V	
<i>Rhizobium japonicum</i> 3.2	6	Low			V	
<i>Mycoplana bullata</i> NCIB 9440	9	64.8			III	
<i>Mycoplana dimorpha</i> NCIB 9439	10	65			III	
" <i>Achromobacter</i> " <i>hartlebii</i> NCIB 8129	11	63.7	12	64.8	III	
" <i>Achromobacter</i> " sp. AB 1196	6	63.1			IV	
" <i>Achromobacter</i> " sp. AB 1293	7	63.1			IV	
" <i>Alcaligenes</i> " sp. AB 940	7	62.8	5	64.1	IV	
<i>Phyllobacterium myrsinacearum</i> LMG ₂ t ₁			7	63.9	IV	
<i>Phyllobacterium myrsinacearum</i> LMG ₂ t ₂			6	63.6	IV	
<i>Phyllobacterium myrsinacearum</i> LMG ₃ t ₁			4	64	IV	
<i>Phyllobacterium myrsinacearum</i> LMG ₃ t ₂			6	64.3	IV	
<i>Phyllobacterium rubiacearum</i> LMG ₁ t ₁			4	64	IV	
" <i>Chromobacterium lividum</i> " NCTC 10590			2	63	V	
" <i>Chromobacterium lividum</i> " NCTC 10591			2	63.4	V	
" <i>Agrobacterium gypsophilae</i> " IPO 280e			1	62.8	V	
" <i>Agrobacterium azotophilum</i> " (Ulloa)			1	63	V	
" <i>Agrobacterium</i> " sp. Sundman 08 = 113			1	63	V	
<i>Acetobacter aceti</i> subsp. <i>aceti</i> Ch 31	2	Low			V	
<i>Acetobacter pasteurianus</i> subsp. <i>estunensis</i> E	2	Low			V	
<i>Alcaligenes faecalis</i> AB 1286	3	Low			V	
<i>Alcaligenes odorans</i> Gilardi 117	3	Low			V	
<i>Achromobacter denitrificans</i> M250	4	Low			V	
<i>Pseudomonas aureofaciens</i> CCEB 518	3	Low			V	
<i>Pseudomonas cichorii</i> NCPPB 906	4	Low			V	
<i>Pseudomonas coronafaciens</i> NCPPB 1328	2	Low			V	
<i>Salmonella typhimurium</i> 1	3	Low			V	
<i>Erwinia lathyri</i> PA	3	Low			V	
<i>Klebsiella pneumoniae</i> N4B	2	Low			V	
<i>Klebsiella rubiacearum</i>	3	Low			V	

^a Part of the data are from De Ley and Tytgat.^b *T*_{m(e)} is expressed in °C.^c "Low" refers to a weakly stable molecular hybrid with *T*_{m(e)} < 62°C.

FIG. 5. Typical differential denaturation curves of duplexes between ^{14}C -labeled DNA from either strain *Agrobacterium tumefaciens* TT111 or B6, with a number of heterologous DNAs. The type of melting curve (see Fig. 5) is given in parentheses. (a) Homologous control: ^{14}C -labeled DNA from *A. tumefaciens* B6 $\times$ DNA from *A. tumefaciens* B6 (type I). (b) Heterologous control: ^{14}C -labeled DNA from *A. tumefaciens* TT111 $\times$ DNA from *A. radiobacter* ATCC 4718 (B6 group) (type II). (c) ^{14}C -labeled DNA from *A. tumefaciens* B6 $\times$ DNA from the misnamed strain "*Azotomonas insolita*" NCIB 9749 (type II). (d) ^{14}C -labeled DNA from *A. tumefaciens* TT111 $\times$ DNA from *Rhizobium leguminosarum* 316C10a (type III). (e) ^{14}C -labeled DNA from *A. tumefaciens* TT111 $\times$ DNA from *Mycoplana bullata* NCIB 9440 (type III). (f) ^{14}C -labeled DNA from *A. tumefaciens* B6 $\times$ DNA from "*Achromobacter*" *hartlebii* NCIB 8129 (type III). (g) ^{14}C -labeled DNA from *A. tumefaciens* B6 $\times$ DNA from *Phyllobacterium myrsinacearum* LMG ϕ_1 (type IV). (h) ^{14}C -labeled DNA from *A. tumefaciens* B6 $\times$ DNA from "*Alcaligenes*" AB 940 (type IV). (i) ^{14}C -labeled DNA from *A. tumefaciens* TT111 $\times$ DNA from *Pseudomonas coronafaciens* NCPPB 1328 (type V).

there was a positive correlation between the $T_{m(e)}$ of the DNA:rRNA hybrids and the overall phenotypic similarity of the genera involved. The percentage of rRNA binding helps to distinguish genera at the same $T_{m(e)}$ level. The phenotypic similarity between *Agrobacterium* and *Rhizobium* is well known. The $T_{m(e)}$ values of

their intergeneric DNA:rRNA hybrids in stringent conditions are high.

Another argument comes from the strains which we received under the erroneous generic names *Achromobacter hartlebii* NCIB 8129 (number 65), *Alcaligenes* AB 940 (number 67), and the presumed agrobacteria AB 1196 and

AB 1293 (numbers 66 and 68). The three AB strains are identical or almost identical because of their high DNA homology (over 90%, unpublished data), and almost identical protein electropherograms (Fig. 6). They have been isolated from pleural fluid (AB 1196), sputum (AB 1293), and spinal fluid (AB 940) in Denmark (H. Lautrop, personal communication); they are probably casual contaminants, rather than causal pathogens. The *Achromobacter hartlebii* organism NCIB 8129 was presumably isolated

in the United States; no further information on its origin was available. On phenotypic grounds these four organisms, in particular strain NCIB 8129, were suspected to be either *Agrobacterium* or closely related to it (36, 42, 72). The *Achromobacter hartlebii* strain was probably for this reason reclassified (wrongly, according to our results) as *Agrobacterium* sp. in the *Catalogue of Strains* (5). All four strains form DNA:rRNA hybrids of high $T_{m(e)}$ with *Agrobacterium*. There is thus a distinct correlation be-

FIG. 6. Electropherograms of soluble proteins from a few reference strains of *Agrobacterium*, *Alcaligenes faecalis*, and *Alcaligenes denitrificans*, several misnamed "Agrobacterium" strains, and taxa related to *Agrobacterium* and/or *Rhizobium*. The method of Kersters and De Ley (41) was followed. Also used were unpublished data from the same authors.

tween $T_{m(e)}$ and phenotypic similarity. Although these four organisms are related to *Agrobacterium*, they definitely are not members of this genus. Their protein electropherograms (Fig. 6) are different from the electropherograms of over 200 authentic agrobacteria (Kerstens and De Ley, unpublished data). They are likewise neither *Alcaligenes* nor *Achromobacter*, because of the type of gluconate metabolism (22) and of their quite different protein electropherograms (Fig. 6). These four strains are far removed from the *Alcaligenes* area in Fig. 3. They must be renamed, but we prefer to wait until more information becomes available.

There are three other genera whose rRNA similarity overlaps with *Agrobacterium* and the peritrichous *Rhizobium*, namely *Azotomonas*, *Mycoplana*, and *Phyllobacterium*. Their taxonomic positions are uncertain; they are not mentioned in *Bergey's Manual of Determinative Bacteriology* (9). The genus name *Azotomonas* was created by Stapp (66) for free-living nitrogen-fixing coccoids and rods from soil, with one to three polar flagella. Neither of the two organisms, *Azotomonas fluorescens* NCIB 9884 and *Azotomonas insolita* NCIB 9749, conforms to this description. They both have two to four peritrichous flagella (15), and they do not fix nitrogen (37, 59). *Azotomonas* is a genus of uncertain status. The position of the strain *Azotomonas insolita* NCIB 9749 in the *Agrobacterium* area (Fig. 3) suggested that it was misnamed. We confirmed by DNA:DNA hybridization (Table 6) and by protein electrophoresis (Fig. 6) that it is indeed an *Agrobacterium* of cluster 1. *Azotomonas fluorescens* NCIB 9884 is not an *Agrobacterium*. The genus name *Mycoplana* was created by Gray and Thornton (31) to include motile branching cells, able to attack phenol. Both species *Mycoplana bullata* NCIB 9440 and *Mycoplana dimorpha* NCIB 9439 were isolated from soil. The genus name *Phyllobacterium* was created by Knösel (45, 46) for bacteria isolated from leaf nodules of

Rubiaceae, *Myrsinaceae*, etc. We used *Phyllobacterium rubiacearum* isolated from *Pavetta zimmermania*, and *Phyllobacterium myrsinacearum* isolated from *Ardisia crispa* and *Ardisia crenata*. The protein electropherograms of each of the five *Phyllobacterium* strains are very similar (Fig. 6), strongly suggesting that they are phenotypically very similar, although they were isolated from three different plant species. The rRNA parameters of the five strains are very close and overlap with these of the peritrichous *Rhizobium*. Knösel (45, 46) located *Phyllobacterium* in the family of the *Rhizobiaceae*.

It is quite remarkable and perhaps not a coincidence that the rRNA features of two misnamed *Chromobacterium lividum* (*Chromobacterium folium*) strains, NCTC 10590 and 10591, are in the vicinity of the *Phyllobacterium* rRNA area. These strains were isolated from germinating seeds of, and are present in, leaf nodules of *Psychotria nairobiensis* and *Ardisia crispa*, respectively (4). They might be related to *Phyllobacterium* (38), but they are not identical with the latter genus (Fig. 3 and 6). Both strains fall quite outside the rRNA area of authentic *Chromobacterium* strains. This was also true when their DNA was hybridized with ¹⁴C-labeled rRNA from the authentic neotype strains of *Chromobacterium violaceum* and *Chromobacterium lividum* (J. De Ley and P. Segers, manuscript in preparation). Our results show that both strains NCTC 10590 and 10591 should be severed from *Chromobacterium*. Several phenotypic differences in support of our genotypic results were collected in Table 7. The appropriate generic name of both *Chromobacterium folium* strains will have to be decided later.

Azotomonas fluorescens NCIB 9884, *Mycoplana*, *Phyllobacterium*, the *Achromobacter hartlebii* organism NCIB 8129, the strains AB 940, AB 1196, AB 1293 (and perhaps the *Chromobacterium folium* organisms) display weak

TABLE 7. Some phenotypic differences between authentic *Chromobacterium lividum* strains and the supposed chromobacteria from leaf nodulated plants^a

Feature	Authentic <i>Chromobacterium lividum</i>	Strains NCTC 10590 and 10591
Mol % G + C	65-72	61 and 63
N ₂ fixation	Not reported	+
Habitat	Common in soil and water in temperate regions	Leaf-nodulated <i>Ardisia crispa</i> , <i>Psychotria nairobiensis</i> , and others
Pigment	Violacein	Nature not determined
Attack on fructose, glucose, galactose, arabinose, xylose, maltose, sorbitol, inositol	Oxidative	No attack

^a Data taken from references 4 and 9.

but distinct DNA similarities with *Agrobacterium* (Table 6), with type III and IV melting curves testifying to their remote relationship to this genus. From all of the above data we conclude that these organisms belong in the family of the *Rhizobiaceae*, together with *Agrobacterium* and *Rhizobium*.

The next lower degree of rRNA relationship consists of the acetic acid bacteria, *Zymomonas* and *Beijerinckia*, with a $\Delta T_{m(e)}$ of about 14°C. It may be significant to note that most organisms with a $T_{m(e)}$ above 65°C are associated with plants. This may point to a common evolutionary origin.

When there are more mismatches in the DNA:rRNA hybrid, and the $T_{m(e)}$ falls below 65°C, the taxonomic distances increase. From hybridizations with ¹⁴C-labeled rRNA from other gram-negative bacteria, it likewise appeared that this temperature is a lower limit for significant taxonomic conclusions, below which all foreign taxa are located together in a confused mass of overlapping rRNA areas (De Ley et al., manuscript in preparation). The instability of the DNA:rRNA hybrids of *Agrobacterium* with the *Enterobacteriaceae*, *Azotobacter*, *Azomonas*, *Pseudomonas*, the *Alcaligenes-Bordetella bronchiseptica* group, *Aeromonas*, *Chromobacterium*, *Bacillus*, *Campylobacter*, *Arthrobacter*, *Corynebacterium*, *Microbacterium*, *Nocardia*, and *Mycobacterium rhodochrous* (Fig. 3 and 4) agrees with the known facts that these taxa are phenotypically very different from *Agrobacterium*. The reverse hybridizations of *Agrobacterium* DNA with ¹⁴C-labeled rRNA from several organisms (Table 5) allow the same conclusion.

Skyring and Quadling (63) suggested, from their principal component analysis of a variety of soil bacteria, that *Arthrobacter* is a rather close relative of *Agrobacterium* and *Rhizobium*. To check this assumption we tested quite a number of coryneforms. The rRNA cistrons of all of them are quite different from *Agrobacterium* and *Rhizobium*. A thorough examination of Skyring's and Quadling's (63) data disclosed that, after the first clustering cycle, quite a number of clusters (for example, 104, 107, 109, 112, etc.) were very heterogeneous and contained many foreign intruders; after the second clustering cycle the groups become even more heterogeneous. This procedure may be very useful to identify soil bacteria, but it seems unsuitable for taxonomic conclusions.

"*Achromobacter*" strains Ruiter 1 and 5 (numbers 69 and 70) cause browning of marinated herring (60). The taxonomic position of these organisms remained uncertain in spite of Ruiter's proposal that they probably belong in

Achromobacter anitratus. We established (De Ley et al., unpublished data) that they belong in the *Enterobacteriaceae*. Their hybrid parameters with *Agrobacterium* ¹⁴C-labeled rRNA fall indeed into this family.

The genus *Agrobacterium* had its share of misnamed strains and species. We checked quite a number of them by rRNA hybridization. One expects that all of these strains will fall outside the *Agrobacterium* area, and they do.

Bacterium gypsophila was isolated from galls on *Gypsophila* plants and described by Brown in 1934 (8). It was renamed *Agrobacterium* by Starr and Weiss (67). There is convincing evidence that these organisms belong in *Erwinia herbicola* (18, 30; De Ley and De Smedt, manuscript in preparation). We used one of Brown's original strains (ATCC 13329) and two strains, IPO 445 and IPO 280e, isolated from roses in the Netherlands in 1969 and 1964, respectively. The DNA:rRNA parameters put these three strains correctly in the *Enterobacteriaceae*. Strain IPO 280e forms no DNA:DNA hybrid with *Agrobacterium* (Table 6). The protein electropherogram of strain ATCC 13329 shows that it is not an *Agrobacterium* (Fig. 6).

Hansen and Smith (34) isolated *Bacterium pseudotsugae* from gall diseases on Douglas fir; this organism was renamed *Agrobacterium* by Săvulescu (61). "*A.*" *pseudotsugae* NCPPB 180 had a DNA quite different from other *agrobacteria* (18). Therefore, its taxonomic position became doubtful. This strain displayed about the same biochemical reactions as *Arthrobacter* (morphology, pathogenicity, etc., were not tested; 64), but this could not be considered as proof of taxonomic identity, because of the clustering techniques (63) as mentioned above. The DNA:rRNA parameters of our two strains of "*Agrobacterium*" *pseudotsugae* (numbers 114 and 115) located them among the coryneforms, in the vicinity of several authentic *Arthrobacter* strains. The protein electropherograms of both strains NCPPB 180 and ATCC 13330 are almost identical to each other and are different from *Agrobacterium* (Fig. 6).

Ahrens (1) and Ahrens and Rheinheimer (2) isolated from the Baltic Sea several bacteria, which they at first classified in seven new species in *Agrobacterium*. Fortunately Ahrens withdrew this proposal (9). We completely agree with this withdrawal: the genus *Agrobacterium* has been under investigation in our laboratory for the last two decades and none of the hundreds of authentic strains from all over the world resemble phenotypically or genotypically any of Ahrens' organisms. The results presented in the present paper again support this conclusion. The DNA:rRNA parameters of

Ahrens' strains do not place them in *Agrobacterium*. "*Agrobacterium*" *agile* A 82 (number 32) has rRNA characteristics close to *Pseudomonas* (J. De Ley and P. De Vos, manuscript in preparation). "*A.*" *ferrugineum* A43 (number 27), and "*A.*" *kielense* B9 (number 28) seem to belong in the vicinity of the peritrichous *Rhizobium*, *Mycoplana*, *Phyllobacterium*, and the AB strains (Fig. 3). It seems to us that both organisms belong in the family of the *Rhizobiaceae*, but their exact taxonomic position is unknown. The remaining strains "*A.*" *luteum* B14, "*A.*" *gelatinovorum* B6, and "*A.*" *aggregatum* B1 (numbers 29, 30, and 31) are much less related. Since Ahrens' strains and *Agrobacterium* require different growth media, no comparable protein electropherograms could be prepared. Our data do not support the tentative suggestion of Hendrie et al. (36) that Ahrens' strains might belong in *Alcaligenes*.

V. Sundman (68, 69) isolated and described several lignanolytic bacteria from Finland, such as strains K17 (NCIB 10467) from decaying sawdust, 08 (NCIB 10469) from decaying brushwood, and M14 (NCIB 10468) from forest soil. These organisms were all tentatively included in *Agrobacterium* (68, 69). In our experience, however, phenotypic features (Kerstens and De Ley, unpublished data), protein electropherograms (Fig. 6), DNA:DNA hybridization (Table 6), and DNA:rRNA hybridization (Fig. 3) excluded these three strains from the latter genus. Strains K17 and 08 are almost identical (Fig. 6). The strains 08 and M14 (numbers 33 and 34) did not fall in the *Agrobacterium* area. Strain 08 cannot be identified from its position in Fig. 3, but we know from other data that both K17 and 08 are taxonomically not far removed from *Alcaligenes* (De Ley and Segers, unpublished data). Strain M14 is even less related to *Agrobacterium* and we have not been able to identify it; we only know it does not belong in the *Rhizobiaceae*, the *Enterobacteriaceae*, the *Vibrionaceae*, the *Pseudomonadaceae*, the *Azotobacteriaceae*, *Alcaligenes*, or *Arthrobacter* (De Ley et al., unpublished data).

Ulloa and Herrera (73) isolated and described bacteria from pozol (fermented maize dough, used as food in Southwest Mexico); one of the strains was called *Agrobacterium azotophilum*. Its ability to fix atmospheric N_2 was stressed (70). The description given by these authors, the position of the strain on the rRNA similarity map (Fig. 3), its lack of DNA homology with *Agrobacterium* (Table 6), and its protein electropherogram (Fig. 6) show, however, that this organism does not belong in *Agrobacterium*.

The approach which we have explored has thus not only taxonomic, but also diagnostic

value: we established that a number of organisms were mislabeled as *Agrobacterium*: (i) the pseudotsugae strains, (ii) the gypsophilae strains, (iii) Ahrens' strains from the Baltic Sea, (iv) Sundman's lignanolytic strains, and (v) Ulloa and Herrera's strain from Mexican pozol.

ACKNOWLEDGMENTS

J.D.L. thanks the Fonds voor Kollektief Fundamenteel Onderzoek for research and personnel grants. J.D.S. is indebted to the same Fonds for a scholarship. We are indebted to all the individuals and institutes who kindly provided strains.

REPRINT REQUESTS

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