Classification of *Rhizomonas suberifaciens*, an Unnamed *Rhizomonas* Species, and *Sphingomonas* spp. in rRNA Superfamily IV

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Thermal melting profiles of hybrids between ³H-labeled rRNA of *Rhizomonas suberifaciens*, the causal agent of corky root of lettuce, and chromosomal DNAs from 27 species of gram-negative bacteria indicated that the genus *Rhizomonas* belongs to superfamily IV of De Ley. On the basis of the melting temperatures of DNA hybrids with rRNAs from the type strains of *R. suberifaciens*, *Sphingomonas paucimobilis*, and *Sphingomonas capsulata*, *Rhizomonas* strains constitute a separate branch in superfamily IV, which is closely related to but separate from branches containing *Zymomonas mobilis*, *Sphingomonas* spp., and *S. capsulata*. *Sphingomonas yanoikuyae* and *Rhizomonas* sp. strain WI4 are located toward the base of the *Rhizomonas* rRNA branch. DNA-DNA hybridization indicated that *S. yanoikuyae* is equidistant from *Rhizomonas* sp. strain WI4 and *S. paucimobilis*. Sequences of 270 bp of 16S ribosomal DNAs from eight strains of *Rhizomonas* spp., eight strains of *Sphingomonas* spp., and *Agrobacterium tumefaciens* indicated that *S. yanoikuyae* and *Rhizomonas* sp. strains WI4 and CA16 are genetically more closely related to *R. suberifaciens* than to *Sphingomonas* spp. Thus, *S. yanoikuyae* may need to be transferred to the genus *Rhizomonas* on the basis of the results of further study.

Rhizomonas suberifaciens was described recently (27) as the causal agent of corky root disease of lettuce in California (24), Florida (5, 23), Wisconsin, and New York (23). At the present time, *R. suberifaciens* is the only species in the genus *Rhizomonas*. However, strains causing corky root of lettuce have been isolated from samples from Wisconsin, California, and The Netherlands, which indicates either that the species is relatively heterogeneous or that other species produce the same disease in lettuce (25, 26, 27).

One of the strains that probably belongs to a different species of the genus *Rhizomonas* is strain WI4, which has a yellow pigment (absent in *R. suberifaciens*), a wider sugar utilization pattern, and a higher G+C content than *R. suberifaciens* (63 mol%, compared with 59 mol% for *R. suberifaciens*) and which exhibits a relatively low level of DNA-DNA hybridization with the type strain of *R. suberifaciens* (27). This strain was considered to be a member of an unnamed *Rhizomonas* species closely related to *Sphingomonas paucimobilis* (formerly *Pseudomonas paucimobilis*) (13, 27, 29). Another strain, strain CA16, which was isolated from lettuce roots in California but was not pathogenic to lettuce, exhibited 70% DNA homology with strain WI4 under moderately stringent conditions and probably belongs to the same *Rhizomonas* species (26). Strains CA16 and WI4 were also grouped into one cluster on the basis of fatty acid profiles (25).

The genus *Sphingomonas* was described at about the same time as the genus *Rhizomonas* with four named species in addition to the type species, *S. paucimobilis* (27, 29). *S. paucimobilis* was originally isolated from human clinical specimens and hospital environments (13) but later was also found in the rhizospheres of plants (2). *R. suberifaciens* and *S. paucimobilis* have many morphological, physiological, and biochemical characteristics in common (13, 27); the most important characteristic is a relatively large proportion

of 2-hydroxylated myristic acid in the whole-cell fatty acid profiles (6, 14, 27, 29). However, there are some important differences between these species. *S. paucimobilis* has a much wider sugar utilization pattern, grows at a higher temperature, and is not pathogenic to lettuce (27). Moreover, the G+C content of *S. paucimobilis* chromosomal DNA is 6 mol% higher than the G+C content of *R. suberifaciens* chromosomal DNA (27). Despite these differences between *S. paucimobilis* and *R. suberifaciens*, it was suggested that *S. paucimobilis* may belong to the genus *Rhizomonas* on the basis of the similarity in fatty acid compositions and the relatively high level of DNA-DNA homology between *S. paucimobilis* and strain CA1 of *R. suberifaciens* (27).

Both *Rhizomonas* and *Sphingomonas* species contain ubiquinone Q10 in their membranes (27). Isoprenoid quinones are characteristic for the higher groupings of bacteria (4). With the exception of the genus *Acetobacter*, which has ubiquinones Q8 and Q9 in addition to ubiquinone Q10 (4), the presence of ubiquinone Q10 as the main isoprenoid quinone seems to be restricted to rRNA superfamily IV (27) as defined by De Ley and coworkers (9-11). As used in this study, rRNA superfamily IV corresponds to the alpha group of the *Proteobacteria* as defined by Stackebrandt et al. (21). Because of the importance that can be attached to quinone analysis, it is likely that *R. suberifaciens* also belongs to superfamily IV (27).

S. paucimobilis and *Sphingomonas capsulata* belong to a single rRNA cluster in rRNA superfamily IV (3). The genus *Zymomonas*, a genus containing ethanol-producing bacteria isolated from palm wine, represents another rRNA branch in this cluster (12). Since *R. suberifaciens* seems to be related to *S. paucimobilis*, these organisms could belong to the same branch or closely related branches of rRNA superfamily IV.

The objectives of this study were to determine whether the genus *Rhizomonas* belongs to superfamily IV and to study the relationships among the genera *Rhizomonas*, *Sphingomonas*, and *Zymomonas*.

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MATERIALS AND METHODS

Bacterial strains. All of the strains which we used are shown in Table 1. *Agromonas oligotrophica* ATCC 43045 was included because it contains ubiquinone Q10 and probably belongs to superfamily IV (19). *Pseudomonas fluorescens* ATCC 13525^T (T = type strain), *Pseudomonas syringae* pv. *syringae* Kado 5D4214, and *Xanthomonas campestris* pv. *campestris* Kado 2D511 were included as representatives of superfamily II, and *Pseudomonas cepacia* Kado 23D4 was included as a representative of superfamily III (11). Strains WI4 and CA16, which are members of an unnamed *Rhizomonas* species distinct from *R. suberifaciens* (26, 27), were included to determine their relationships with *S. paucimobilis* and *S. capsulata*.

Most strains were cultured in S medium broth (24) for 2 days at 28°C with shaking. Cultures of *R. suberifaciens* and *Agromonas oligotrophica* strains were grown for 4 days and 1 week, respectively. Cultures of strains assigned to the genera *Sphingomonas* and *Zymomonas* were grown either on S medium or as described previously (9, 12). *Beijerinckia indica* ATCC 9039 was cultured in American Type Culture Collection (ATCC) medium 13 (1), *Xanthobacter autotrophicus* ATCC 35674 was cultured in nutrient broth, and *Gluconobacter oxydans* subsp. *sphaericus* ATCC 33280 was grown in ATCC medium 1.

DNA-rRNA hybridization. Thermal melting profiles were determined for hybrids between ³H-labeled 16S and 23S rRNAs from *R. suberifaciens* CA1^T, *S. paucimobilis* LMG 1227^T, and *S. capsulata* LMG 2830^T and DNAs from the strains listed in Table 1.

DNA was isolated as described by van Bruggen et al. (27) and was applied to nitrocellulose filters (diameter, 47 mm; type 11358; Sartorius) under a vacuum (8). Filters containing DNA were stored under a vacuum at 4°C. To calculate the size of disc needed for hybridization (50 µg of DNA per disc), 10-mm-diameter discs were punched out, incubated overnight at 50°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0) containing 20% formamide as a mock hybridization, rinsed in 2× SSC, and dried. The DNA was released from the test discs in 1.6 M HClO₄ by using the method of Meijs and Schilperoort (17) and was quantified as described by Richards (20).

Cultures of *S. capsulata* LMG 2830^T and *S. paucimobilis* LMG 1227^T were labeled in vivo by adding [¹⁴C]orotate and [³H]adenine, respectively. rRNA was isolated as described by Moore and McCarthy (18). Final purification of rRNA was performed as described by De Ley and De Smedt (7). *R. suberifaciens* CA1^T was labeled in vivo with 5 µCi of [5,6-³H]uridine (35 to 50 Ci/mmol) per ml at the beginning of the log phase and twice during exponential growth. Cultures were given unlabeled uridine (40 µmol) 2 h before harvesting. RNA was extracted by using the method of Ausubel et al. (1a) and was resuspended in DNase buffer (20 mM Tris-Cl, 10 mM MgCl₂; pH 8.0). Residual DNA was removed by treatment with DNase (300 U/ml) at 37°C for 30 min. The RNA was then extracted once with phenol-chloroform (equilibrated in 10 mM Tris-Cl [pH 8.0]–1 mM EDTA) and twice with chloroform and then precipitated with 0.04 volume of 5.0 M NaCl and 2.5 volumes of absolute ethanol. Crude RNA was resuspended in gradient buffer (0.1 M sodium acetate buffer [pH 5.0] containing 0.1 M NaCl, 2.0 mM EDTA, and 0.2% diethylpyrocarbonate). Sucrose gradients (10 to 40% [wt/vol] sucrose in gradient buffer) were loaded with 0.5 to 1.0 mg of RNA and centrifuged at 109,000 × g for 17 h at 4°C. Fractions were collected and measured

for radioactivity, and the fractions containing 23S and 16S RNAs were dialyzed against an excess of 2× SSC at 4°C and stored at –20°C. Fractions containing 23S and 16S RNAs were identified by electrophoresis on a 1.0% agarose gel in 1× MOPS buffer (20 mM MOPS [morpholinepropanesulfonic acid], 5.0 mM sodium acetate, 2.0 mM EDTA) against known 23S and 16S rRNA standards. The specific activity of the rRNA was approximately 18,000 dpm/µg.

The conditions used for hybridization of rRNA to DNA on filter discs were those described by De Smedt and De Ley (9). Unhybridized rRNA was removed by digesting it with RNase A at 37°C for 1 h. The RNase was prepared by dissolving 100 mg of RNase A (code RAF, from bovine pancreas; Worthington Biochemical Corp., Freehold, N.J.) in a beaker containing 75 ml of water, 1.753 g of NaCl, and 0.630 g of citric acid (pH 3.0), boiling the resulting preparation for 5 min, and chilling it on ice. At room temperature, the pH was adjusted to 7.0 with NaOH, and the volume brought up to 100 ml with preboiled water. The concentration of RNase used was determined in a preliminary hybridization experiment performed with a reference strain for which the DNA-rRNA melting temperature was known. After the unhybridized rRNA was removed, discs were rinsed twice in 50 ml of 2× SSC at room temperature. Each disc was put through a temperature gradient consisting of 5°C increments from 50 to 90°C in 2 ml of 1.5× SSC–20% formamide in scintillation vials (9). A 10-ml portion of scintillation cocktail (Bio-Safe; Research Products Corp., Mount Prospect, Ill.) was added to each vial, and the amount of rRNA released at each temperature was calculated from the radioactive counts determined by liquid scintillation counting for 20 min with a model LS 7500 instrument (Beckman Instruments, Inc., Fullerton, Calif.).

Melting profiles were determined for each hybrid at least four times with rRNA from *R. suberifaciens* CA1^T and two times with rRNAs from *S. capsulata* LMG 2830^T and *S. paucimobilis* LMG 1227^T. Melting temperatures were defined as the temperatures at which 50% of the radioactivity was removed from the filters. A dendrogram was constructed for the *Rhizomonas*, *Sphingomonas*, and *Zymomonas* species on the basis of the melting temperatures for hybrids with rRNAs from *R. suberifaciens*, *S. capsulata*, *S. paucimobilis*, and *Zymomonas mobilis* (12).

rDNA sequencing. DNAs corresponding to 16S rRNAs (rDNAs) of all of the *Rhizomonas* and *Sphingomonas* strains mentioned above were amplified by the polymerase chain reaction (PCR), using a model 480 DNA thermal cycler (Perkin Elmer Corp., Norwalk, Conn.). For comparison, rDNA of *Agrobacterium tumefaciens* was also included. Chromosomal DNA was extracted as described by van Bruggen et al. (27) and was quantified spectrophotometrically at 260 nm. Two oligodeoxynucleotides with sequences similar to those of conserved regions of 16S rRNA at positions 9 to 29 (oligo 6) (14a) and positions 519 to 536 (primer A) (15) of *Escherichia coli* (28) were used as PCR primers. Oligo 6 (5'-GAGTTTGATCCTGGCTCAGGA) was synthesized by the DNA Synthesis Facility, Department of Biochemistry, University of California, Berkeley. Primer A (5'-TATTACCGCGGCTGCTG) was obtained from Operon Technologies, Inc., Alameda, Calif. The PCR was carried out in a reaction volume of 33 µl containing 1 to 2 ng of bacterial DNA, 1 µmol of each primer, 1 U of *Taq* polymerase (Promega Corp., Madison, Wis.), 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris (pH 8.3), and dideoxynucleotide triphosphates at concentrations of 200 µM. A total of 25 amplification cycles were used. The PCR conditions con-

sisted of denaturation at 95°C for 1.5 min, primer annealing at 55°C for 2 min, and chain extension at 72°C for 2 min. Amplified DNA was purified from agarose gels with Gene Clean (Bio 101, Inc., La Jolla, Calif.), precipitated with ethanol-sodium acetate (100% ethanol-3 M sodium acetate-water, 950:40:10), and resuspended in sterile TE (10 mM Tris-HCl, 1.0 mM EDTA; pH 8.0).

Sequencing reactions were carried out with the ds DNA Cycle Sequencing System (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.) according to the manufacturer's instructions, using the PCR-amplified DNA as the template and oligo 6 as the sequencing primer. The PCR sequencing program consisted of 20 cycles of denaturation at 95°C for 45 s, primer annealing at 55°C for 5 s, and chain extension at 72°C for 30 s. Reaction products were electrophoresed in a 6% polyacrylamide gel containing 8 M urea in 1× TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.004 M EDTA). Sequencing gels were dried on filter paper at 80°C under a vacuum and were exposed to X-ray film.

Sequence data were aligned and pairwise similarities were calculated by using the GCG program (Genetics Computer Group, University of Wisconsin, Madison) on a VAX main-frame computer. Phylogenetically conserved positions were deleted, and variable region sequences were subjected to cluster analysis by using the parsimony and bootstrapping methods (MacIntosh version 3.0L of PAUP [22]). *Agrobacterium tumefaciens* was used as the outgroup organism.

DNA-DNA hybridization. DNAs from *R. suberifaciens* CA1^T, *Rhizomonas* sp. strain WI4, and *S. paucimobilis* ATCC 29837^T were hybridized with DNAs from five strains of *R. suberifaciens*, *Rhizomonas* sp. strain WI4, and nine strains of *Sphingomonas* spp.

Chromosomal DNA was extracted as described by van Bruggen et al. (27). DNA was quantified spectrophotometrically at 260 nm. DNA from each strain was denatured in 0.4 M NaOH-10 mM EDTA, and 1.0-μg portions were transferred onto Zeta-probe membranes (Bio-Rad Laboratories, Richmond, Calif.) or Hybond N⁺ membranes (Amersham, Arlington Heights, Ill.) with a slot blot vacuum manifold (model Minifold II; Schleicher and Schuell, Keene, N.H.). The blots were washed with 0.4 M NaOH and rinsed in 2× SSC as described previously (27), air dried, and fixed at 80°C in a vacuum oven for 1 to 2 h. DNA from *R. suberifaciens* CA1^T, *Rhizomonas* sp. strain WI4, or *S. paucimobilis* ATCC 29837^T was denatured in a boiling water bath for 5 min and labeled with [³²P]dCTP by using an oligolabeling kit (Random Primed DNA Labeling Kit; Boehringer Mannheim). Unincorporated nucleotides were removed by passing the mixture through Sephadex G-50 filters. Slot blots were prehybridized overnight (27). Denatured, labeled probe was added to the blots, and the preparations were hybridized for 15 to 20 h at 50°C (moderately stringent conditions). The blots were washed and rinsed as described previously (27), blotted, and autoradiographed with type XAR5 film (Kodak, Rochester, N.Y.). The extent of hybridization was determined by directly measuring the counts per minute on the blots, using a Radioanalytic Imaging System (Ambis Systems, Inc., San Diego, Calif.). There were three replications for each strain randomized on several blots.

RESULTS

The melting temperatures of hybrids between *R. suberifaciens* CA1^T rRNA and DNAs from *R. suberifaciens* strains ranged between 78 and 80.5°C (Table 1). Hybrids with *S.*

yanoikuyae DNA melted at 75°C, and hybrids with *Rhizomonas* sp. strain WI4, *Z. mobilis*, *S. paucimobilis*, and *S. capsulata* DNAs melted at 70 to 72°C. The melting temperatures of hybrids with DNAs from other members of superfamily IV ranged from 63 to 66°C. The melting temperatures of hybrids with DNA from *Agromonas oligotrophica*, which was not classified in a superfamily but contained ubiquinone Q10, fell in the same range as the melting temperatures of hybrids with DNAs from strains belonging to superfamily IV. The melting temperatures of hybrids with DNAs from strains belonging to superfamilies II and III ranged from 59 to 61°C (Table 1).

Hybrids of *S. paucimobilis* rRNA with DNAs from *S. paucimobilis*, *S. parapaucimobilis*, and *Sphingomonas* genospecies I and II had melting temperatures between 79 and 80.6°C (Table 1), while hybrids with DNAs from *S. capsulata* and *Sphingomonas adhaesiva* melted at 71.1 and 75.2°C, respectively. The melting temperatures of hybrids with DNAs from *Z. mobilis* and *Rhizomonas* spp. were similar to the melting temperatures of hybrids with *S. capsulata* DNA. For hybrids of rRNA from *S. capsulata*, the melting temperatures were 80°C with *S. capsulata* DNA and between 71 and 73°C with DNAs from strains belonging to other *Sphingomonas* spp., *Z. mobilis*, and *Rhizomonas* spp.

The results are shown in a melting temperature dendrogram in Fig. 1. The following four closely related but separate rRNA branches in superfamily IV can be distinguished: one branch for the genus *Zymomonas*, one branch for *S. capsulata*, one branch for other *Sphingomonas* spp., and one branch for *R. suberifaciens* and *S. yanoikuyae*. *Rhizomonas* sp. strain WI4 is located at the base of the *Rhizomonas* branch.

DNA hybridization with DNAs from *R. suberifaciens* CA1^T, *Rhizomonas* sp. strain WI4, and *S. paucimobilis* ATCC 29837^T confirmed that strains FL2, FL3, NY12, and WI3 belong to the genus *Rhizomonas*, while strain WI4 is as closely related to the genus *Rhizomonas* as to the genus *Sphingomonas* (Table 2). *S. yanoikuyae* exhibited higher levels of DNA hybridization with *Rhizomonas* sp. strain WI4 and *S. paucimobilis* ATCC 29837^T than with *R. suberifaciens* CA1^T. The levels of DNA hybridization with *S. paucimobilis* were higher for *Sphingomonas* genospecies I and II, *Sphingomonas parapaucimobilis*, and *S. adhaesiva* than for *S. capsulata*.

rDNA sequencing with primer oligo 6 resulted in a 270-base sequence (Fig. 2), corresponding to positions 50 to 350 of *Escherichia coli* rRNA (28). Compared with *E. coli* rRNA, 30 bases were deleted. The 270-base sequences displayed enough variability to clearly distinguish three main clusters, one for *S. capsulata*, one for all other species of the genus *Sphingomonas* except *S. yanoikuyae*, and one for *R. suberifaciens*, *Rhizomonas* sp. strains WI4 and CA16, and *S. yanoikuyae* (Fig. 2 and 3). One base in particular, corresponding to position 52 of *E. coli* rRNA in a relatively conserved region, helped to separate the last three strains in a subgroup of the *Rhizomonas* cluster (Fig. 3).

DISCUSSION

The melting temperatures of hybrids between *R. suberifaciens* CA1^T rRNA and DNAs from various strains belonging to superfamilies II, III, and IV indicated that *R. suberifaciens* is a member of superfamily IV. Within this superfamily, strains of *R. suberifaciens* constitute a separate branch which is closely related to the rRNA branch contain-

ing *Zymomonas* spp. and the rRNA branch containing *Sphingomonas* spp., which includes *S. paucimobilis*, *S. parapaucimobilis*, *S. adhaesiva*, and two *Sphingomonas* genospecies (29). Both the rRNA-DNA hybridization data and the rDNA sequences of *S. capsulata* showed that *S. capsulata* does not belong to the *Sphingomonas* rRNA branch and needs to be transferred to a new genus.

A close relationship between the genera *Rhizomonas* and *Sphingomonas* was postulated previously on the basis of similar fatty acid compositions, various physiological test results, and relatively high levels of DNA-DNA hybridization between *R. suberifaciens* CA1^T and *S. paucimobilis* under moderately stringent conditions (27). At that time, we considered the possibility of transferring *S. paucimobilis* (then *P. paucimobilis*) to the newly proposed genus *Rhizomonas* (27). However, the levels of hybridization between

FIG. 1. Melting temperature [$T_{m(e)}$] dendrogram showing the *Zymomonas*, *S. capsulata*, *S. paucimobilis*, and *R. suberifaciens* rRNA branches in rRNA superfamily IV.

TABLE 1. Bacterial strains used in this study and mean melting temperatures for hybrids between chromosomal DNAs from the strains and rRNAs from *R. suberifaciens* CA1^T, *S. paucimobilis* LMG 1227^T, and *S. capsulata* LMG 2830^T

Super-family	Taxon	Strain	Source ^a	Mean G+C content (mol%)	Melting temp (°C) for hybrid with rRNA from:		
					<i>R. suberifaciens</i> CA1 ^T	<i>S. paucimobilis</i> LMG 1227 ^T	<i>S. capsulata</i> LMG 2830 ^T
IV	<i>R. suberifaciens</i>	NY12 (= LMG 9835)	van Bruggen	59.4	80.5		
		FL2 (= LMG 9831)	van Bruggen	57.9	80.0	71.2	72.4
		CA1 ^T (= LMG 9830 ^T)	van Bruggen	58.0	79.6	71.2	73.0
		FL3 (= LMG 9832)	van Bruggen	59.4	78.1	70.4	71.7
		W13 (= LMG 9837)	van Bruggen	58.9	78.0		
	<i>S. yanoikuyae</i>	LMG 11252 ^T (= CCUG 28380 ^T)	CCUG	63.7	75.3	72.2	
	<i>Rhizomonas</i> sp.	W14 (= LMG 11032)	van Bruggen	62.9	72.4	70.7	72.6
	<i>S. capsulata</i>	LMG 2830 ^T (= ATCC 14666 ^T)	LMG	65.9		71.1	79.8
		ATCC 14666 ^T	ATCC		71.5		
	<i>S. paucimobilis</i>	LMG 1227 ^T (= ATCC 29837 ^T)	LMG	65.4		80.6	72.4
		ATCC 29837 ^T	ATCC		71.1		
		LMG 10922 ^T (= CCUG 27290 ^T)	CCUG	67.9	71.9	75.2	
	<i>S. adhaesiva</i>	LMG 10923 ^T (= CCUG 27291 ^T)	CCUG	66.9	72.0	79.5	
	<i>S. parapaucimobilis</i>	LMG 10925 (= CCUG 27654)	CCUG	66.2	70.3	79.7	
	<i>Sphingomonas</i> genospecies I	LMG 10924 (= CCUG 27293)	CCUG	65.2	70.5	79.0	
	<i>Sphingomonas</i> genospecies II						
	<i>Z. mobilis</i> subsp. <i>mobilis</i>	LMG 405 ^T	LMG		70.8	70.5	
		LMG 445	LMG	48.8			72.8
	<i>Z. mobilis</i> subsp. <i>pomaceae</i>	LMG 448 ^T	LMG	48.2	70.1	69.3	
	<i>Ochrobactrum anthropi</i>	NCTC 12168 ^T	NCTC	57.2	66.4		
	<i>Beijerinckia indica</i>	ATCC 9039 ^T	ATCC	56.4	66.0		67.2
	<i>Rhizobium meliloti</i>	8D15	Kado		65.5		
	<i>Xanthobacter autotrophicus</i>	ATCC 35674	ATCC	67.3	65.5		66.5
	<i>Azorhizobium caulinodans</i>	ATCC 43989	ATCC		65.3		
	<i>Pseudomonas diminuta</i>	501	Baumann		65.2		
		LMG 1793 ^T	LMG	65.9			62.9
	<i>Gluconobacter oxydans</i> subsp. <i>sphaericus</i>	ATCC 33280	ATCC	56.3	64.4		62.9
	<i>Agrobacterium tumefaciens</i>	C-58 (= LMG 287)	Kado		64.0		
	<i>Phyllobacterium myrsinacearum</i>	ATCC 43590	ATCC	60.3	63.7		67.3
	<i>Agrobacterium rhizogenes</i>	3D1 (= ICPB TR108)	Kado		63.4		
	<i>Bradyrhizobium japonicum</i>	ATCC 10324 ^T (= LMG 6138 ^T)	ATCC	64.0	63.4		
	<i>Aquaspirillum itersonii</i>	NCIB 9017	NCIB	61.8			65.8
	<i>Agromonas oligotrophica</i>	ATCC 43045	ATCC	65.0	63.2	64.5	
	<i>Pseudomonas fluorescens</i> biotype A	11D42 ^T (= ATCC 13525 ^T)	Kado		60.7		
II	<i>Xanthomonas campestris</i> pv. <i>campestris</i>	2D511	Kado		60.6		
	<i>Pseudomonas syringae</i> pv. <i>syringae</i>	5D4214	Kado		60.2		
	<i>Pseudomonas cepacia</i>	23D4	Kado		59.1		

^a ATCC, American Type Culture Collection, Rockville, Md.; Baumann, P. Baumann, Department of Microbiology, University of California, Davis; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; Kado, C. I. Kado, Department of Plant Pathology, University of California, Davis; LMG, Collection of the Laboratory of Microbiology, University of Ghent, Ghent, Belgium; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, London, England; van Bruggen, A. H. C. van Bruggen, Department of Plant Pathology, University of California, Davis.

TABLE 2. Mean percentages of DNA homology between chromosomal DNAs from various strains of gram-negative bacteria and DNAs from *R. suberifaciens* CA1^T, *Rhizomonas* sp. strain WI4, and *S. paucimobilis* ATCC 29837^T

Taxon	Strain	% of DNA homology with DNA from:		
		<i>R. suberifaciens</i> CA1 ^T	<i>Rhizomonas</i> sp. strain WI4	<i>S. paucimobilis</i> ATCC 29837 ^T
<i>R. suberifaciens</i>	CA1 ^T	99.9	9.7	9.4
	NY12	69.3	7.8	8.9
	FL2	75.5	8.3	8.8
	FL3	62.9	7.6	8.4
	WI3	74.4	8.3	7.7
<i>Rhizomonas</i> sp.	WI4	10.8	100.0	12.8
<i>S. adhaesiva</i>	LMG 10922 ^T	4.9	10.0	32.6
<i>S. capsulata</i>	LMG 2830 ^T	6.9	7.4	17.8
	ATCC 14666 ^T	2.3	5.1	12.0
<i>Sphingomonas</i> genospecies I	LMG 10925	4.9	9.1	51.2
<i>Sphingomonas</i> genospecies II	LMG 10924	4.5	9.4	51.3
<i>S. parapaucimobilis</i>	LMG 10923 ^T	5.1	7.1	59.3
<i>S. paucimobilis</i>	LMG 1227 ^T	4.9	8.5	114.0
	ATCC 29837 ^T	3.7	6.5	96.8
<i>S. yanoikuyae</i>	LMG 11252 ^T	5.9	16.1	23.3

DNA from *R. suberifaciens* CA1^T and DNA from *Rhizomonas* sp. strain WI4 or *S. paucimobilis* were much lower in the present study than reported previously (27), despite similar hybridization conditions. This difference proved to be due to a change in the method used for quantifying the radioactive signals (a radioanalytic imaging system instead of densitometry of autoradiographed blots) (13a, 26). The low levels of DNA hybridization between *R. suberifaciens* and *S. paucimobilis* and the differences in the melting temperatures of DNA-rRNA hybrids and in the rDNA sequences in this

study showed that the members of the *S. paucimobilis* rRNA branch and the members of the *Rhizomonas* rRNA branch differ enough to warrant two separate genera. Nevertheless, within superfamily IV, these genera are very closely related and differ from other genera in this superfamily primarily in that their outer membranes contain 2-hydroxylated myristic acid rather than the 3-hydroxylated fatty acids that occur in other gram-negative bacteria (14, 27).

The melting temperatures of hybrids between *Rhizomonas* sp. strain WI4 DNA and *R. suberifaciens* strain CA1^T rRNA

<u>At</u>	AACACATGCA	AGTCGAACG.	.CCC...CG	CAAGGAGTGG	CAGACGGGTG	AGTAACGCGT	GGGAACATAC
<u>Sc</u>	AACACATGCA	AGTCGAACGA	ACCCTT..CG	GGGTTAGTGG	CGCACGGGTG	CGTAACGCGT	GGGAATCTGC
<u>Sp</u>	AACACATGCA	AGTCGAACGA	AGGCTT..CG	GCCTTAGTGG	CGCACGGGTG	CGTAACGCGT	GGGAATCTGC
<u>Rs</u>	AACACATGCA	AGTCGAACGA	GACCTT..CG	CGTCTAGTGG	CGCACGGGTG	CGTAACGCGT	GGGAATCTAC
<u>WI4</u>	AATACATGCA	AGTCGAACGA	TCCCTT..CG	GGGATAGTGG	CGCACGGGTG	CGTAACGCGT	GGGAATCTGC
<u>Sy</u>	AATACATGCA	AGTCGAACGA	GATCTT..CG	GATCTAGTGG	CGCACGGGTG	CGTAACGCGT	GGGAATCTGC
<u>At</u>	CCTTTCTGTC	GGAATAGCTC	CGGGAAACTG	GAATTAATAC	CGCATACGCC	CTACGGGGGA	AAGATTTATC
<u>Sc</u>	CCTTTGCTTC	GGAATAACAG	TTAGAAATGA	CTGCTAATAC	CGGATGATGA	CTTCGGTCCA	AAGATTTATC
<u>Sp</u>	CCTTAGGTTT	GGAATAACAG	CTGGAAACGC	CTGCTAATAC	CGGATGATAT	CGCGAGATCA	AAGATTTATC
<u>Rs</u>	CCTTGGGTTT	GGAATAACAG	TTAGAAATGA	CTGCTAATAC	CGGATGATGA	CGAAAGTCCA	AAGATTTATC
<u>WI4</u>	CCTTGGGTTT	GGAATAACGT	CGGGAAACTG	ACGCTAATAC	CGGATGATGA	CGTAAGTCCA	AAGATTTATC
<u>Sy</u>	CCTTGGGTTT	GGAATAACTT	CTGGAAACGG	AAGCTAATAC	CGGATGATGA	CGTAAGTCCA	AAGATTTATC
<u>At</u>	GGGGAAGGAT	TGGCCCGCGT	TGGATTAGCT	AGTTGGTGGG	GTAAAGGC.T	ACCAAGGCCA	CGATCCATAG
<u>Sc</u>	GGCAAAGGAT	GAGCCCGCGT	AGGATTAGGT	AGTTGGTGGG	GTAAAGGCCT	ACCAAGCCGA	CGATCCTTAG
<u>Sp</u>	GCCTGAGGAT	GAGCCCGTGT	TGGATTAGGT	AGTTGGTGGG	GTAAAGGCCT	ACCAAGCCGA	CGATCCATAG
<u>Rs</u>	GCCCAAGGAT	GAGCCCGCGT	AGGATTAGCT	AGTTGGTGGG	GTAAAGGCCT	ACCAAGCCGA	CGATCCTTAG
<u>WI4</u>	GCCCAAGGAT	GAGCCCGCGT	AGGATTAGCT	AGTTGGTGGG	GTAAAGGCCT	ACCAAGCCGA	CGATCCTTAG
<u>Sy</u>	GCCCAAGGAT	GAGCCCGCGT	AGGATTAGCT	AGTTGGTGGG	GTAAAGGCCT	ACCAAGCCGA	CGATCCTTAG
<u>At</u>	CTGGTCTGAG	AGGATGATCA	GCCACATTGG	GACTGAGACA	CGGCC.AAAC	TC.TACGGAG	
<u>Sc</u>	CTGGTCTGAG	AGGATGATCA	GCCACACTGG	GACTGAGACA	CGGCCAGAC	TCCTACGGAG	
<u>Sp</u>	CTGGTCT.AG	AGGATGATCA	GCCACACTGG	GACTGAGACA	CGGCCAGAC	TCCTACGGAG	
<u>Rs</u>	CTGGTCTGAG	A.GATGATCA	GCCACACT.G	GACTGAGACA	C.G.CCAGAC	TC.TACGG.G	
<u>WI4</u>	CTGGTCTGAG	AGGATCATCA	GCCACACTGG	GACTGAGACA	CG.CCCAGAC	TCCTACGGAG	
<u>Sy</u>	CTGGTCTGAG	AGGATGATCA	GCCACACTGG	GACTGAGACA	CGGCCAGAC	TCCTACGGAG	

FIG. 2. rDNA sequences for *Agrobacterium tumefaciens* C-58 (At), *S. capsulata* LMG 2830^T (Sc), *S. paucimobilis* LMG 1227^T (Sp), *R. suberifaciens* CA1^T (Rs), *Rhizomonas* sp. strain WI4 (WI4), and *S. yanoikuyae* LMG 11252^T (Sy), corresponding to positions 50 to 350 of *E. coli* rRNA.

FIG. 3. Dendrogram determined by parsimony and bootstrapping, using 270-bp rDNA sequences (Fig. 2). The data are derived from *R. suberifaciens* CA1^T, FL2, FL3, NY12, and WI3, *Rhizomonas* sp. strains WI4 and CA16, *S. yanoikuyae* LMG 11252^T, *Sphingomonas* genospecies I strain LMG 10925, *Sphingomonas* genospecies II strain LMG 10924, *S. paucimobilis* LMG 1227^T and ATCC 29837^T, *S. adhaesiva* LMG 10922^T, *S. parapaucimobilis* LMG 10923^T, and *S. capsulata* LMG 2830^T and ATCC 14666^T. *Agrobacterium tumefaciens* C-58 was used as an outgroup.

were similar to the melting temperatures of hybrids between DNAs from *Sphingomonas* strains and strain CA1^T rRNA. This indicates that, genetically, strain WI4 is as far removed from *R. suberifaciens* as it is from *Sphingomonas* species. However, additional hybridizations with rRNAs from *S. capsulata* LMG 2830^T and *S. paucimobilis* LMG 1227^T showed that strain WI4 does not belong to the rRNA branches of these species. Although the melting temperatures of DNA-rRNA hybrids indicate that strain WI4 could occupy a separate position equidistant from the genus *Sphingomonas* and *R. suberifaciens*, a phylogenetic analysis of rDNA sequences suggested that strain WI4 is more closely related to *R. suberifaciens* than to *Sphingomonas* species. Final classification of strain WI4 will require more polyphasic studies that include other closely related isolates.

On the basis of the melting temperature of the hybrid between *S. yanoikuyae* LMG 11252^T DNA and *R. suberifaciens* CA1^T rRNA (75.3°C), *S. yanoikuyae* belongs on the *R. suberifaciens* rRNA branch and genotypically can be considered a member of the genus *Rhizomonas*. This was confirmed by the results of a phylogenetic analysis of rDNA

sequences. The sequences of *S. yanoikuyae* rRNA were more similar to the sequences of *Rhizomonas* sp. strain WI4 and CA16 rRNAs than to the sequences of rRNAs from *R. suberifaciens* strains. However, the levels of DNA-DNA hybridization with *R. suberifaciens* CA1^T and *Rhizomonas* sp. strain WI4 were not high enough to consider *S. yanoikuyae* a member of either of these *Rhizomonas* taxa. More genetic and physiological studies will be needed to classify *S. yanoikuyae* as a species of the genus *Rhizomonas*.

This was the first study which showed that rDNA sequences corresponding to positions 50 through 350 of *E. coli* rRNA (28) could be used to separate strains of the genus *Rhizomonas* from *Sphingomonas* strains and strains of a potentially new genus containing *S. capsulata*. Moreover, these sequences contained enough variability to distinguish among several *Rhizomonas* and *Sphingomonas* species. Yabuuchi et al. (29) used rRNA sequences corresponding to positions 1220 through 1376 of *E. coli* 16S rRNA with primer C of Lane et al. (15) to initiate the sequences. A phylogenetic analysis of those sequences resulted in good separation of *S. paucimobilis*, *S. adhaesiva*, *S. yanoikuyae*, and *S. capsulata*, but not of *S. paucimobilis*, *S. parapaucimobilis*, and *Sphingomonas* genospecies I and II (29). These last four species had slightly different rDNA sequences in our study. The grouping obtained by Yabuuchi et al. (29) generally agreed with our grouping except for the placement of *S. capsulata* and *S. yanoikuyae* strains; on the basis of our results, these strains may belong to an unnamed genus and the genus *Rhizomonas*, respectively. If *Rhizomonas* strains had been included in the study of Yabuuchi et al., *S. yanoikuyae* might have clustered in the *Rhizomonas* group. Additional phylogenetic, biochemical, and physiological studies are currently under way to characterize and describe several new *Rhizomonas* species (25a).

ACKNOWLEDGMENTS

We thank C. I. Kado and P. Baumann for supplying bacterial cultures. We are very grateful to B. Kirkpatrick for advice on sequencing of rDNA and for supplying primer oligo 6.

Most of this work was supported by the California Iceberg Lettuce Research Board. M.G. is indebted to the National Fund for Medical Scientific Research, Belgium, for research and personnel grants.

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