Phylogenetic Evidence for *Sphingomonas* and *Rhizomonas* as Nonphotosynthetic Members of the Alpha-4 Subclass of the *Proteobacteria*

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To clarify the taxonomic relationships of the genera *Rhizomonas* and *Sphingomonas*, the 16S rRNA sequence of *Rhizomonas suberifaciens* IFO 15211^T (T = type strain) was determined. A phylogenetic analysis of aligned 16S rRNA gene sequences revealed that eight species of the genus *Sphingomonas* and *R. suberifaciens* are closely related to *Erythrobacter longus* and *Porphyrobacter neustonensis* and, therefore, belong in the alpha-4 subclass of the *Proteobacteria*. Within this subclass, *Sphingomonas* species and *R. suberifaciens* are phylogenetically interrelated and comprise several subgroups. Our findings show that the genus and species definitions of these organisms are in need of revision.

The genus *Sphingomonas*, whose type species, *Sphingomonas paucimobilis*, was previously named *Pseudomonas paucimobilis* Holmes et al. (6), was proposed by Yabuuchi et al. (42) for yellow-pigmented, motile rods with single polar flagella and nonmotile, nonfermentative, gram-negative rods. *Sphingomonas paucimobilis* contains large amounts of a unique sphingoglycolipid with the long-chain base dihydrosphingosin, 2-hydroxymyristic acid (41, 43), and isoprenoid quinone Q-10. Five species, *Sphingomonas paucimobilis*, *Sphingomonas parapaucimobilis*, *Sphingomonas yanoikuyae*, *Sphingomonas adhaesiva*, and *Sphingomonas capsulata*, were described by Yabuuchi et al. (42). Recently, we described three new species of the genus *Sphingomonas*, *Sphingomonas sanguis*, *Sphingomonas macrogoltabidus*, and *Sphingomonas terrae* (29). *Sphingomonas sanguis* is the new species name proposed for *Sphingomonas* genospecies 1 of Yabuuchi et al. (42). *Sphingomonas macrogoltabidus* and *Sphingomonas terrae* are polyethylene glycol-utilizing bacteria (11, 12) which were previously classified as *Flavobacterium* species. Polyethylene glycol 4000 is utilized by a single bacterium, *Sphingomonas terrae*, but polyethylene glycol 6000 is utilized by symbiotic mixed cultures of two strains (13), and the dominant bacterium in the mixed cultures is *Sphingomonas macrogoltabidus*.

Recently, we compared the 16S rRNA sequences of the eight previously described and newly proposed species of the genus *Sphingomonas* with the 16S rRNA sequences of 15 representative species belonging to the alpha subclass of the *Proteobacteria* (29), and we found that the eight *Sphingomonas* species form a large and heterogeneous cluster which is clearly separate from all other representatives of the alpha subclass of the *Proteobacteria* (26, 38) except *Erythrobacter longus* (23, 24).

On the other hand, the genus *Rhizomonas* and the single species *Rhizomonas suberifaciens* were proposed by van Bruggen et al. (32) for the gram-negative, motile, rod-shaped bacteria that cause corky root of lettuce. Most strains of *Rhizomonas suberifaciens* are oligotrophic (31), but the morphological, physiological, and chemotaxonomic characteristics

of these organisms are similar to those reported for *Sphingomonas paucimobilis* (33, 42). Neither *Rhizomonas suberifaciens* nor *Sphingomonas paucimobilis* produces acid on peptone-glucose medium, but both organisms produce acid on ammonium or nitrate-glucose medium (6). Strains of these two species have the same isoprenoid quinone (ubiquinone 10) and similar fatty acid compositions (8, 17, 33), and the results of grouping based on these two characteristics have been shown to coincide with the results of grouping based on rRNA-DNA homology data (33).

On the basis of these findings, we suggested previously that species belonging to the genera *Sphingomonas* and *Rhizomonas* are closely related to each other (29).

Recently, on the basis of sequencing data for 270 bases of 16S rRNA genes from eight strains of *Rhizomonas* species and eight strains of *Sphingomonas* species, van Bruggen et al. (33) reported that *Sphingomonas yanoikuyae* is more closely related to *Rhizomonas suberifaciens* than to other *Sphingomonas* species and should be transferred to the genus *Rhizomonas* and that *Sphingomonas capsulata* should be removed from the genus *Sphingomonas* and placed in a separate genus.

In order to investigate the phylogenetic interrelationships of these organisms, we determined the 16S rRNA gene sequence and chemotaxonomic characteristics of *Rhizomonas suberifaciens* and compared the resulting data with data for *Sphingomonas* species, *Erythrobacter longus*, and other members of the alpha subclass of the *Proteobacteria*.

In this paper we describe the 16S rRNA sequence of *Rhizomonas suberifaciens* and phylogenetic evidence which indicates that the genera *Sphingomonas* and *Rhizomonas* are nonphotosynthetic members of the alpha-4 subclass of the *Proteobacteria*.

MATERIALS AND METHODS

Cultures. *Rhizomonas suberifaciens* IFO 15211^T (= ATCC 49355^T = NCPPB 3629^T) (T = type strain) and IFO 15212 (= ATCC 49382 = NCPPB 3631) were cultured at 28°C in shake flasks containing (per liter) 5.0 g of peptone, 2.5 g of glucose, 1.3 g of K₂HPO₄, 0.5 g of MgSO₄ · 7H₂O, 0.5 g of KNO₃, and 0.06 g of Ca(NO₃)₂ · 4H₂O (pH 7.2) (PG medium). *Erythrobacter longus* IFO 14126^T (= ATCC 33941^T) was cultivated in seawater medium containing (per liter) 4.0 g of peptone, 2.0

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TABLE 1. Comparison of morphological, physiological, and biochemical characteristics of the genera *Sphingomonas* and *Rhizomonas*

Characteristic	<i>Sphingomonas</i> ^a	<i>Rhizomonas</i>
Plant pathogenicity	— ^b	+ ^c
Oligotroph	—	v ^c
Morphological characteristics		
Cell shape	Rods	Rods
Color of colonies	Yellow or whitish yellow	Yellow or whitish yellow
Motility	v	v
Flagellum	Single, polar	Single, lateral, subpolar, or polar
Physiological characteristics		
Oxidase	+	+
Catalase	+	+
Acid produced from glucose	+	+
Arginine dihydrolase	—	—
Nitrate reduction	v	+
Bacteriochlorophyll <i>a</i>	—	—
Chemical characteristics		
Isoprenoid quinone	Q-10	Q-10
Major cellular fatty acids		
Nonpolar fatty acid	18:1	18:1
2-Hydroxy fatty acids	14:0, 15:0, 16:0	14:0, 15:0
3-Hydroxy fatty acid	—	—
Sphingolipids	+ (d-18:0, d-19:1, d-20:1, d-21:1)	+ (d-18:0, d-20:1 d-21:1)
LPS	—	— ^d
G+C content (mol%)	61.6–67.8	58.0–63.0

^a Data from references 29 and 42.^b +, all strains are positive; —, all strains are negative; v, variable from strain to strain.^c Data from reference 33.^d All strains are positive according to van Bruggen et al. (33).

g of yeast extract, 10 mg of FeSO₄ · 7H₂O, 750 ml of seawater, and enough distilled water to bring the volume up to 1,000 ml (23, 24).

Cellular lipids and fatty acid analysis. Cells were harvested after they were cultured for 24 h in PG medium and freeze-dried, and then 50 mg of dried cells was mixed with 2 ml of 5% HCl in methanol and the preparation was heated at 100°C for 3 h. Fatty acid methyl esters were extracted with *n*-hexane and were separated by thin-layer chromatography by using an *n*-hexane-diethyl ether (1:1, vol/vol) solvent system. Nonpolar acids and 2-hydroxy and 3-hydroxy fatty acids, visualized by spraying plates 0.02% dichlorofluorescein in ethanol, were extracted with diethyl ether and analyzed by gas-liquid chromatography as described previously (29). The long-chain bases of the cellular sphingolipids were obtained from acid hydrolysates of dried cells as described by Yano et al. (44). The bases were then subjected to thin-layer chromatography by using a of chloroform-methanol-water (65:25:4, vol/vol) solvent system

and analyzed as trimethylsilyl ethers by gas chromatography-mass spectrometry.

Analysis of bacteriochlorophyll. Bacteriochlorophyll was extracted from dried cells with methanol by the method of Stal et al. (28). Samples were extracted twice for 2 h, and the two extracts were pooled in the dark at room temperature and purified by thin-layer chromatography by using a petroleum ether-acetone-methanol (82:16:2, vol/vol) solvent system.

In vitro amplification of rRNA genes. DNA was used for in vitro amplification of the rRNA gene by the PCR technique (19) in combination with a 16S rRNA gene-specific primer pair, 5'-AGTTTGATCCTGGCTC OH-3' (identical to sequence positions 10 to 25 in the *Escherichia coli* numbering system [1]) and 5'-AAGGAGGTGATCCAGCC OH-3' (complementary to positions 1541 to 1525), as described previously (29). Amplification was carried out by using a *Taq* polymerase kit (Cetus Inc.). The PCR cycle parameters were as follows: preheating for 2.5 min at 95°C, denaturation for 1 min at 94°C,

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1 AGUUUGAUCCUGGCUCAGAACGACGUGGCGGCAUGCCUAAACACAUGCAAGUCGAACGAGACCUUCGGGUCUAGUGGCGCACGGGUGCGUAAACGCGUGG
101 GAAUCUACCCUUGGGUUCGGAUAAACAGUUAAGAAUGACUGCUAAUACCGGAUGAUGACGAAAGUCCAAAGAUUUUACGCCCAAGGAGAGCCCGCGUAG
201 GAUUAAGCUAGUUGGUGAGGUAAAGGCUCACCAAGGCGACGAUCCUAGCUGGUCUGAGAGGAUGAUCAGCCACACUGGGACUGAGACACGGCCAGACUC
301 CUACGGGAGGCAGCAGUGGGGAUUAUUGGACAUGGGCGAAAAGCCUGAUCCAGCAUUGCCGCGUGAGUGAUGAAGGCCUUAAGGGUUGUAAAGCUCUUUA
401 CCCGGGAUGAUAAUGACAGUACCGGGAGAAUAGCUCGCGCUAACUCCGUGCCAGCAGCCGCGUAAUACGGAGGGAGCUAGCGUUGUUCGGAUUAUCUG
501 GCGUAAAGCGCACGUAGGCGGCUAUUCAAGUCAGAGGUGAAAGCCCGGGGCUAACCCCGGAUAGCCUUGAAACUAGAUAAGCUAGAAUCUGGGAGAG
601 GUGAGUGGAAUUCGAGUGUAGAGGUGAAUUCGUAUAUUCGGAAGAACACCAAGUGGCGAAGGCGGCUACUGGACCAGAAUUGACGUCUGAGGUGCGA
701 AAGCUGGGGAGCAACAGGAUUAAGAUACCCUGGUAGUCCACGCCGUAACCAUGAUAAUAGCUGUCUGGGUUCUUAAGACUUAAGGUGGCGCACGUAAC
801 GCAUUAAGUUAUCCGCCUGGGGAGUACGGUUGCAAGAUAUAAACUCUAAAGGAAUUGACGGGGGCGUCACAAGCGGUGGAGCAUGUGGUUUAUUCGAAG
901 CAACGCGCAGAACCUCAGCGUUGACAUCUCCGCAUUCUCCAGAGAUUGGAUUCUUCAGUUCGGCUGGAUGAGACAGGUGUCUGCAUGGCGU
1001 CGUCAGCUCGUGUCGUGAGAUUUGGGUUAAGUCCCGCAACGAGCGCAACCCUCCGCUUUAAGUUAACCAUUAUAGUUGGGUACUCUAAAGGAACCGCGG
1101 GUGAUAAAGCCGGAGGAAGGUGGGGAUGACGUAAGUCCUUAUGGCCUUAACGCGCUGGGUACACACGUGCUACAAGGCGGUGACAGUGGGCAGCUAAC
1201 UCGCGAGAGUAUGCUAAUCUCUAAAAACCGUCUAGUUCGGAUUGUUCUCUGCAACUCGAGAGCAUGAAGGCGGAUUCGCUAGUAAUUCGCGGAUCAGCAU
1301 GCCGCGGUGAAUACGUUCCAGGCCUUGUACACACCGCCGUCACACCAUGGGAGUUGGAUUCACCGAAGGCGCUGAGCUAACCCGCAAGGGAGGCGAGG
1401 GGACCACGGUGGGU

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FIG. 1. Partial 16S rRNA sequence of *Rhizomonas suberifaciens*. The first and last nucleotides are analogous to positions 10 and 1483 of the *Escherichia coli* sequence (1).

annealing for 2.5 min at 58°C, and extension for 2.5 min at 72°C. After 30 cycles, the final step was incubation for 5 min at 72°C. The lengths of the amplified fragments were determined by agarose (1%, wt/vol) gel electrophoresis.

Sequence determination and analysis. The amplified DNA was purified by using Suprec-01 (Takara Co., Ltd., Shiga, Japan) after electrophoresis on an agarose (1%, wt/vol) gel. The purified DNA was sequenced by using a Sequenase kit for ³⁵S-dATP (United Biochemical Inc.) and the following primers: 5'-AGTTTGATCCTGGCTC OH-3' (identical to positions 10 to 25), 5'-GTGTTACTCACCCGT OH-3' (complementary to positions 123 to 109), 5'-TACGGGAGGCAGC AG OH-3' (identical to positions 343 to 357), 5'-CTGTGC CTCCCGTAG OH-3' (complementary to positions 357 to 342), 5'-GTGCCAGCAGCCGCGG OH-3' (identical to positions 515 to 530), 5'-ACCGGGCTGCTGGC OH-3' (complementary to positions 531 to 517), 5'-TCTACGCATTTC ACC OH-3' (complementary to positions 704 to 690), 5'-GTC AATTCCTTTGAGTTT OH-3' (complementary to positions 924 to 907), 5'-AGGGTTGCGCTCGTTG OH-3' (complementary to positions 1115 to 1100), 5'-CCATTGTAGCACGT GT OH-3' (complementary to positions 1242 to 1227), 5'-AC GGGCGGTGTGTAC OH-3' (complementary to positions 1406 to 1392), and 5'-GGCTACCTTGTTACGA OH-3' (complementary to positions 1510 to 1495). DNA sequences were aligned by using the ODEN system (7). Nucleotide substitution rates (K_{nuc} values) were calculated from all of the available sequence data after alignment (14), and a phylogenetic tree was constructed by the neighbor-joining method (20). The topology of the trees was evaluated by performing a bootstrap analysis of the sequence data, using Clustal V (5).

Nucleotide sequence accession numbers. The sequences were aligned with previously published sequences obtained from the DNA Data Bank of Japan, GenBank, and EMBL data bases and deposited under the following accession numbers: *Escherichia coli*, M25588; *Sphingomonas paucimobilis*, D13725; *Sphingomonas parapaucimobilis*, D13724; *Sphingomonas sanguis*, D13726; *Sphingomonas terrae*, D13727; *Sphingomonas yanoikuyae*, D13728; *Sphingomonas adhaesiva*, D13722; *Sphingomonas capsulata*, M59296; *Sphingomonas macrogoltabidus*, D13723; *Erythrobacter longus*, M96744; *Porphyrobacter neustonensis*, M96745; *Roseobacter denitrificans*, M96746; *Afipia felis*, M65248; *Agrobacterium tumefaciens*, M11223; *Ancylobacter aquaticus*, M62790; *Magnetospirillum magnetotacticum*, M58171; *Azospirillum lipoferum*, M59061; *Bartonella bacilliformis*, M65249; *Beijerinckia indica*, M59060; *Blastobacter denitrificans*, X66025; *Bradyrhizobium japonicum*, X66024; *Brucella abortus*, X13695; *Caulobacter bacteroides*, M83796; *Cowdria ruminantium*, X61659; *Ehrlichia risticii*, M21290; *Erythrobacter longus*, M59062; *Hirschia baltica*, X52909; *Hyphomicrobium vulgare*, X53182; *Hyphomonas jannaschiana*, M83806; *Methylobacterium organophilum*, M29028; *Methylosinus trichosporium*, M29024; *Paracoccus denitrificans*, X69159; *Porphyrobacter neustonensis*, M96745; *Pseudomonas diminuta*, M59064; *Rhodobacter capsulatus*, M34129; *Rhodomi-*

crobiu vanniellii, M34127; *Rhodopila globiformis*, M59066; *Rhodospirillum rubrum*, M32030; *Rickettsia prowazekii*, M21789; *Rochalimaea quintana*, M11927; *Roseobacter denitrificans*, M96746; *Simonsiella muelleri*, M59071; and *Wolbachia pipientis*, X61768. The nucleotide sequence data for *Rhizomonas suberifaciens* have been deposited in the DNA Data Bank of Japan data base under accession number D13737.

RESULTS AND DISCUSSION

Chemotaxonomic characteristics. Gas chromatography and gas chromatography-mass spectrometry analyses of trimethylsilyl derivatives of long-chain bases from *Rhizomonas suberifaciens* IFO 15211^T and *Erythrobacter longus* IFO 14126^T revealed that dihydrosphingosin d-20:1 was the major component and that d-18:0, d-19:1, and d-21:1 also were present. *Erythrobacter longus* is a bacteriochlorophyll-containing bacterium, although it does not have photosynthetic activity. We examined whether species of the genus *Sphingomonas* contain bacteriochlorophyll and confirmed that bacteriochlorophyll was absent in *Sphingomonas paucimobilis* IFO 14135^T, *Sphingomonas capsulata* IFO 12533^T, and *Rhizomonas suberifaciens* IFO 15211^T. Thus, we suggest that the presence of bacteriochlorophyll is a distinctive characteristic which distinguishes the genus *Erythrobacter* from both the genus *Sphingomonas* and the genus *Rhizomonas*. Table 1 shows the morphological, physiological, biochemical, and chemotaxonomic characteristics of the genera *Sphingomonas* and *Rhizomonas*, and on the basis of these data the two taxa are indistinguishable. All of the strains of both genera were characterized by having isoprenoid quinone Q-10, by having 2-hydroxymyristic acid, and by lacking 3-hydroxy fatty acid. The G+C content of the DNA of *Rhizomonas suberifaciens* IFO 15211^T was 58.0 to 63.0 mol%, and the G+C contents of strains of the genus *Sphingomonas* were 61.6 to 67.8 mol%.

In general, gram-negative bacteria contain lipopolysaccharide (LPS) (endotoxin) and 3-hydroxy C₁₀ to C₁₈ fatty acids in their outer membranes. Although 3-OH fatty acids were not detected in *Rhizomonas suberifaciens*, the presence of LPS in this species has been reported by van Bruggen et al. (31, 32). Kawahara et al. reported that *Sphingomonas paucimobilis* lacks the usual LPS in the outer membrane (10) but contains amphiphilic glycosphingolipid, which might play a role similar to the role of LPS in other gram-negative bacteria (9). In view of these contradictory results, the presence of LPS in *Rhizomonas* species should be reexamined.

Phylogenetic analyses. To clarify the phylogenetic relationships of the species belonging to the genera *Sphingomonas* and *Rhizomonas* and *Erythrobacter longus*, we determined the 16S rRNA sequence of *Rhizomonas suberifaciens* IFO 15211^T (Fig. 1) and compared it with the sequences of eight species belonging to the genus *Sphingomonas* (29), the sequences of the bacteriochlorophyll-containing bacteria *Porphyrobacter neustonensis* (4) and *Roseobacter denitrificans* (22), and the sequences of 30 other species belonging to the alpha subclass

FIG. 2. Unrooted phylogenetic tree showing the relationships of *Rhizomonas suberifaciens* IFO 15211^T, *Sphingomonas* species, and other members of the alpha subclass of the *Proteobacteria*. The following reference microorganisms were included: alpha-1 group species *Rhodopila globiformis* (30, 40), *Magnetospirillum magnetotacticum* (2), and *Rhodospirillum rubrum* (30, 40); alpha-2 group species *Hyphomicrobium vulgare* (25), *Rhodomicrobium vanniellii* (40), *Ancylobacter aquaticus* (39), *Afipia felis* (37), *Blastobacter denitrificans* (37), *Bradyrhizobium japonicum* (37), *Methylosinus trichosporium* (30), *Methylobacterium organophilum* (30), *Agrobacterium tumefaciens* (30, 40), *Bartonella bacilliformis* (18), *Rochalimaea quintana* (36), *Caulobacter bacteroides* (27), and *Pseudomonas diminuta* (30); alpha-3 group species *Hyphomonas jannaschiana* (25), *Hirschia baltica* (21), *Paracoccus denitrificans* (15, 40), *Rhodobacter capsulatus* (30, 40), and *Roseobacter denitrificans* (4); alpha-4 group species *Erythrobacter longus* (40) and *Porphyrobacter neustonensis* (4); alpha group species *Cowdria ruminantium* (3), *Ehrlichia risticii* (34, 35), *Rickettsia prowazekii* (35), and *Wolbachia pipientis* (16); delta-3 group species *Escherichia coli* (30). Bar = 0.01 K_{nuc} unit.

of the *Proteobacteria*. Figure 2 shows an unrooted phylogenetic tree derived from K_{nuc} values that were calculated by using 941 bases from 41 species. Table 2 shows the K_{nuc} and sequence similarity values for *Sphingomonas* species, *Rhizomonas suberifaciens*, and other members of the *Proteobacteria*. As shown in Fig. 2, the branching pattern indicates that *Rhizomonas suberifaciens* and all of the *Sphingomonas* species belong in the alpha-4 subclass of the *Proteobacteria*. Moreover, an analysis of our data confirmed the existence of four major subgroups. The first subgroup consists of *Sphingomonas paucimobilis*, *Sphingomonas parapaucimobilis*, and *Sphingomonas sanguis* (similarity values, 98.3 to 99.3%); the second subgroup comprises the polyethylene glycol-utilizing bacteria *Sphingomonas macrogoltabidus*, *Sphingomonas terrae*, and *Sphingomonas adhaesiva* (similarity values, 97.9 to 98.9%); the third subgroup consists of *Sphingomonas yanoikuyae* and *Rhizomonas suberifaciens* (similarity value, 96.2%); and the last subgroup contains *Sphingomonas capsulata*, which is clearly distinct from other species belonging to the genus *Sphingomonas* (similarity values, 92.2 to 94.8%) and *Rhizomonas suberifaciens* (similarity value, 94.6%).

On the basis of the results described above, it is clear that all of the species belonging to the genera *Sphingomonas* and *Rhizomonas* are nonphotosynthetic members of the alpha-4 subclass of the *Proteobacteria*, but these organisms are phylogenetically interrelated and can be divided into several subgroups. The chemotaxonomic characteristics of the two genera are rather similar, and there are few other remarkable differences in morphological and physiological characteristics which distinguish these subgroups.

van Bruggen et al. (33) suggested that *Sphingomonas yanoikuyae* should be placed in the genus *Rhizomonas* and that *Sphingomonas capsulata* should be transferred to a new genus. Our results supported these conclusions. In addition, six other species of the genus *Sphingomonas* could be divided into two subgroups, each of corresponded to a genus; one subgroup contained the original *Sphingomonas* species (*Sphingomonas paucimobilis*, *Sphingomonas parapaucimobilis*, and *Sphingomonas sanguis*), and the other contained *Sphingomonas adhaesiva*, *Sphingomonas terrae*, and *Sphingomonas macrogoltabidus*.

However, it is not appropriate to transfer these subgroups to other genera at the present time because of a lack of differential phenotypic characteristics. Thus, a thorough revision of the generic and species classification of this group is inevitable.

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