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Phylogenetic structure of the genera *Flexibacter*, *Flexithrix*, and *Microscilla* deduced from 16S rRNA sequence analysis

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The 16S rDNA sequences of 40 strains of 17 species in the genus *Flexibacter*, 5 strains of 4 species in the genus *Microscilla*, and 1 strain of *Flexithrix dorotheae*, including all type strains of approved and validated species in these genera, were determined to reveal their phylogenetic relationships. The 16S rRNA sequence analysis demonstrated the extreme heterogeneity of the genera *Flexibacter* and *Microscilla*. The strains examined diverged into 24 distinct lines of descent (1 group included both flexibacteria and flexithrix, and 1 group included both flexibacteria and microscilla) that were remote from each other at the genus level or higher. *Flexibacter* strains were scattered across the cytophaga-flavobacteria-bacteroides phylum and divided into 20 phylogenetic groups, and the genus *Microscilla* was separated into 5 groups. *Flexibacter flexilis*, the type species of the genus *Flexibacter*, and *Microscilla marina*, the type species of the genus *Microscilla*, were isolated from other organisms in their respective genera. This means that each genus should be restricted to only the type species. *Flexithrix dorotheae*, the type species of the genus *Flexithrix*, clustered with *Flexibacter aggregans*. The heterogeneity was found not only within genera but also within species. *Flexibacter aggregans*, *Flexibacter aurantiacus*, *Flexibacter flexilis*, *Flexibacter roseolus*, *Flexibacter tractuosus*, and “*Microscilla sericea*” each contained phylogenetically distant strains. The taxonomic concept of the genera *Flexibacter*, *Flexithrix*, and *Microscilla* should be reorganized in accordance with the natural relationships revealed in this study.

Key Words—cytophaga-flavobacterium-bacteroides phylum; diversity of flexibacteria; *Flexibacter*; *Flexithrix*; *Microscilla*

Introduction

The genera *Flexibacter*, *Flexithrix*, and *Microscilla* belong to the cytophaga-flavobacteria-bacteroides (cfb) phylum, one of the main phyla of the domain Bac-

teria, together with the genera *Cytophaga*, *Flavobacterium*, and other genera including anaerobes such as *Bacteroides* (Woese, 1987). The taxonomy of the genera *Cytophaga*, *Flavobacterium*, *Flexibacter*, *Flexithrix*, and *Microscilla* has a turbulent history. These genera have been called the *Flavobacterium-Cytophaga* complex, because they share similar phenotypic characteristics (Callies and Mannheim, 1978; Christensen, 1977; Hayes, 1977; Oyaizu and Komagata, 1981; Shewan and McMeekin, 1983).

The genus *Flexibacter* was created by Soriano (1945) to encompass five species of gliding bacteria and the genus *Microscilla* was created by Pringsheim

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(1951) to encompass three species. From the beginning, it was difficult to differentiate the two genera from each other and all original strains of both genera have been lost. Lewin (1969) reisolated morphologically similar organisms and redefined both genera by classifying marine organisms with longer filaments (20–100 μm) in the genus *Microscilla*. This definition was not followed in Bergey's Manual of Determinative Bacteriology, 8th ed. (Leadbetter, 1974), and the genus *Microscilla* was included in the genus *Flexibacter*. Later, Reichenbach (1989b,c) tried to restrict the genus *Microscilla* to marine organisms with G+C contents of the DNA of above 37 mol%. However, he has also stated that too little was known about these bacteria, that these genera were still heterogeneous, and that future research including modern molecular taxonomy was a prerequisite.

The genus *Flexithrix* described by Lewin (1970) accommodates only one species with a single strain. It is a marine organism and may produce sheathed filaments that differentiate it from *Microscilla*. It was also suggested that *Flexithrix* resembles *Flexibacter aggregans* ("*Microscilla aggregans*") (Reichenbach, 1989a). Phylogenetic analyses based on the 16S rRNA sequences have also shown a huge degree of biological diversity in some of these bacteria (Gherna and Woese, 1992; Nakagawa and Yamasato, 1993, 1996).

The understanding and acceptance of the diversity of the *Flavobacterium-Cytophaga* complex have led to emendation and creation of some genera to reclassify those organisms. The genus *Cytophaga* was emended to restrict it to only two species, *Cytophaga hutchinsonii* and *Cytophaga aurantiaca* (Nakagawa and Yamasato, 1996); and *Flavobacterium* was emended to include *Flavobacterium aquatile* and its phylogenetic relatives (Bernardet et al., 1996). Some misnamed cytophagas were allocated to new genera, *Cellulophaga*, *Flammeovirga*, *Marinilabilia*, *Pedobacter*, *Persicobacter*, and *Tenacibaculum* (Bowman, 2000; Johansen et al., 1999; Nakagawa et al., 1997; Nakagawa and Yamasato, 1996; Steyn et al., 1998; Suzuki et al., 1999, 2001); and some misnamed flavobacteria were classified into *Chryseobacterium*, *Empedobacter*, *Myroides*, *Psychroflexus*, *Salegentibacter*, and *Sphingobacterium* (Bowman et al., 1997, 1998; McCammon and Bowman, 2000; Vancanneyt et al., 1996; Vandamme et al., 1994; Yabuuchi et al., 1983).

While the taxonomic confusions in the genera *Cytophaga* and *Flavobacterium* have thus largely been

settled, comprehensive taxonomic studies on the genera *Flexibacter*, *Flexithrix*, and *Microscilla* have not been done yet. In addition, many new taxa belonging to the cfb phylum have been described in the last decade, but the relationships among these new genera, and *Flexibacter*, *Flexithrix*, and *Microscilla* remain unknown. This paper reports phylogenetic studies on the genera *Flexibacter*, *Flexithrix*, and *Microscilla* and discusses the taxonomic structures of these genera deduced from 16S rRNA sequences analyses.

Materials and Methods

Bacterial strains and cultivation. Forty strains of 17 species in the genus *Flexibacter*, 5 strains of 4 species in the genus *Microscilla*, and 1 strain of *Flexithrix dorotheae* were used in this study (Table 1). The type strains of all approved and validated species in these genera were included. The strains were cultivated in the medium recommended by culture collections.

PCR amplification and sequencing of 16S rDNA. The DNA for PCR (Saiki et al., 1988) templates was prepared according to Nakagawa et al. (2001) or by using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). The almost complete 16S rRNA gene (16S rDNA) was amplified by PCR with the primers 9F (5'-GAGTTTGA-TCCTGGCTCAG, the same sequence as positions 9 to 27 [*Escherichia coli* numbering system of Brosius et al. (1978)]) and 1541R (5'-AAGGAGGTGATCCAGCC, complementary to positions 1525 to 1541). The 1.5-kb amplified 16S rDNA fragment was purified by using a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ, USA) or by agarose gel electrophoresis and a QIAquick Gel Extraction Kit (Qiagen). The PCR products were directly sequenced by using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) or a DYEnamic ET Terminator Cycle Sequencing Ready Reaction Kit (Amersham Biosciences) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The oligonucleotide primers used to sequence the full length of amplified 16S rDNA were 9F, 339F (5'-CTCCTACGGGAGGCAGCAG, the same sequence as positions 339 to 357), 785F (5'-G-GATTAGATACCCTGGTAGTC, same as positions 785 to 805), 1099F (5'-GCAACGAGCGCAACCC, same as positions 1099 to 1114), 536R (5'-GTATTACCGCGG-CTGCTG, complementary to positions 519 to 536), 802R (5'-TACCAGGGTATCTAATCC, complementary

Table 1. Strains used in this study.

Species	Latest classification	IFO no.	Obtained from:	Accession no.	Group ^a
<i>Flexibacter aggregans</i>		15973	NCIMB 1389	AB078037	22
<i>Flexibacter aggregans</i>		15974	NCIMB 1391	AB078038	20
<i>Flexibacter aggregans</i>		15975	NCIMB 1432	AB078039	1
<i>Flexibacter aggregans</i>		15976 ^T	NCIMB 1443	AB078040	22
<i>Flexibacter aggregans</i>		15990	NCIMB 1464	AB078041	22
" <i>Flexibacter aggregans</i> subsp. <i>cataliticus</i> "		15977 ^T	NCIMB 1418	AB078042	5
<i>Flexibacter aurantiacus</i>	<i>Flavobacterium johnsoniae</i>	15970 ^T	DSM 6792	AB078043	4
" <i>Flexibacter aurantiacus</i> subsp. <i>copepodarum</i> "		15978 ^T	NCIMB 1394	AB078044	3
" <i>Flexibacter aurantiacus</i> subsp. <i>excathedrus</i> "		16024 ^T	ATCC 23086	AB078045	4
<i>Flexibacter canadensis</i>		15130 ^T	ATCC 29591	AB078046	7
<i>Flexibacter columnaris</i>	<i>Flavobacterium columnare</i>	15943 ^T	IAM 14301	AB078047	4
<i>Flexibacter elegans</i>		15055 ^T	ATCC 23112	AB078048	8
<i>Flexibacter filiformis</i>		15056 ^T	DSM 527	AB078049	6
<i>Flexibacter flexilis</i>		15060 ^T	ATCC 23079	AB078050	11
<i>Flexibacter flexilis</i>		16025	ATCC 23080	AB078051	11
<i>Flexibacter flexilis</i>		16026	ATCC 23089	AB078052	11
<i>Flexibacter flexilis</i>		16027	ATCC 23096	AB078053	11
" <i>Flexibacter flexilis</i> subsp. <i>pelliculosus</i> "		16028 ^T	ATCC 23098	AB078054	12
<i>Flexibacter japonensis</i>		16041 ^T	JCM 9735	AB078055	6
<i>Flexibacter litoralis</i>		15988 ^T	DSM 6794	AB078056	13
<i>Flexibacter maritimus</i>	<i>Tenacibaculum maritimum</i>	15946 ^T	IAM 14317	AB078057	3
<i>Flexibacter ovolyticus</i>	<i>Tenacibaculum ovolyticum</i>	15947 ^T	IAM 14318	AB078058	3
<i>Flexibacter polymorphus</i>		16703 ^T	DSM 9678	AB078059	15
<i>Flexibacter psychrophilus</i>	<i>Flavobacterium psychrophilum</i>	15942 ^T	IAM 14308	AB078060	4
<i>Flexibacter roseolus</i>		16030	ATCC 23087	AB078061	14
<i>Flexibacter roseolus</i>		16486	NCIMB 1433	AB078062	8
<i>Flexibacter roseolus</i>		16707 ^T	DSM 9546	AB078063	16
<i>Flexibacter ruber</i>		16675	LMG 13508	AB078064	8
<i>Flexibacter ruber</i>		16677 ^T	LMG 13857	AB078065	10
<i>Flexibacter sancti</i>		15057 ^T	DSM 784	AB078066	6
<i>Flexibacter sancti</i>		16033	ATCC 23090	AB078067	6
<i>Flexibacter sancti</i>		16034	ATCC 23097	AB078068	6
<i>Flexibacter tractuosus</i>		15979	NCIMB 1393	AB078069	17
<i>Flexibacter tractuosus</i>		15980	NCIMB 1416	AB078070	2
<i>Flexibacter tractuosus</i>		15981	NCIMB 1429	AB078071	17
<i>Flexibacter tractuosus</i>		15989 ^T	DSM 4126	AB078072	17
<i>Flexibacter tractuosus</i>		16035	ATCC 23151	AB078073	19
<i>Flexibacter tractuosus</i>		16037	ATCC 23145	AB078074	24
<i>Flexibacter tractuosus</i>		16038	ATCC 23191	AB078075	24
<i>Flexibacter tractuosus</i>		16482	NCIMB 12854	AB078076	19
<i>Flexithrix dorotheae</i>		15987 ^T	DSM 6795	AB078077	22
" <i>Microscilla arenaria</i> "		15982 ^T	NCIMB 1413	AB078078	23
" <i>Microscilla furvescens</i> "		15994 ^T	NCIMB 1419	AB078079	18
<i>Microscilla marina</i>		16560 ^T	NCIMB 1400	AB078080	9
" <i>Microscilla sericea</i> "		15983 ^T	NCIMB 1403	AB078081	17
" <i>Microscilla sericea</i> "		16561	NCIMB 1392	AB078082	21

^a Phylogenetic groups recognized by 16S rRNA sequence analysis.

Abbreviations: ^T, Type strain; ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG, BCCMTM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; IAM, IAM Culture Collection, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Osaka, Japan; NCIMB, The National Collections of Industrial and Marine Bacteria, Ltd., Aberdeen, Scotland, UK.

Fig. 1. Phylogenetic tree of *Flexibacter*, *Flexithrix*, and *Microscilla* strains constructed from 16S rRNA sequence analysis.

Scale bar=0.02 K_{nuc} in nucleotide sequences. The numbers on the branches are the confidence limits estimated by bootstrap analysis with 1,000 resamplings. Low values (<500) are not shown. Accession numbers of the sequences of the reference organisms are shown in parenthesis. *Agrobacterium tumefaciens*, *Bacillus subtilis*, and *Escherichia coli* were used as the root organisms.

to positions 785 to 802), 1115R (5'-AGGGTTGCGCT-CGTTG, complementary to positions 1100 to 1115) and 1541R.

Phylogenetic analysis. The 16S rDNA sequences were assembled with the AutoAssembler program (Applied Biosystems). The almost complete 16S rDNA sequences determined for the 46 strains were aligned with the sequences of reference organisms derived from databases by using the CLUSTAL W ver. 1.8 software package (Thompson et al., 1994). The alignments were modified manually against the 16S rRNA secondary structure of *Escherichia coli* (Gutell et al., 1994). Phylogenetic relationships were inferred from the phylogenetic tree based on the neighbor-joining method (Saitou and Nei, 1987) and the K_{nuc} values (Kimura, 1980) by using CLUSTAL W. Positions at which secondary structures varied between strains and caused an ambiguous alignment (142–220, 447–487, 841–845, 991–1045 and 1134–1140), positions before 111 and after 1376, and all sites for which sequences were not determined in any reference organisms were excluded from the analysis. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1,000 replicates.

Nucleotide sequence accession number. The 16S rDNA sequence data determined in this study were deposited in the DDBJ database and appear in the DDBJ, EMBL, GSDB, and NCBI nucleotide sequence databases under accession numbers shown in Table 1. The accession numbers of reference organisms used in phylogenetic analysis are shown in Fig. 1.

Results and Discussion

The almost complete 16S rDNA sequences ranging from position 28 to 1524 on the *Escherichia coli* numbering system were determined for 40 strains of 17 species in the genus *Flexibacter*, 5 strains of 4 species in the genus *Microscilla*, and 1 strain of *Flexithrix dorotheae*. The phylogenetic tree and the similarity matrix based on 16S rRNA sequences are shown in Fig. 1 and Table 2, respectively. It is known that the anaerobes (the genus *Bacteroides* and its relatives) constitute a distinct cluster in the cfb phylum (Paster et al., 1985). None of the strains examined in this study showed close relationships with *Bacteroides* or its relatives. Figure 1 shows that the genera *Flexibacter* and *Microscilla* are extremely heterogeneous. *Flexibacter*

strains are scattered across the phylum and divided into 20 phylogenetic groups. The genus *Microscilla* was separated into 5 groups. Overall, the strains examined were divided into 24 groups (1 group contained both flexibacteria and flexithrix, and 1 group contained both flexibacteria and microscilla) that were remote from each other at genus level or higher. The similarity values of 16S rDNA sequences between each group were lower than 95% (Table 2), although we compared only the conserved region of 16S rDNA (see MATERIALS AND METHODS). Among the 24 groups, groups 1–4 belonged to the family *Flavobacteriaceae* that was proposed and emended recently by Bernardet et al. (1996, 2002). We have shown that the respiratory quinone composition correlated with phylogenetic relationships in the cfb phylum, and all strains in this family examined so far were characterized by possessing menaquinone-6 (MK-6) (Nakagawa and Yamasato, 1993, 1996). Respiratory quinone composition of groups 1 and 2 is unknown. Its analysis is needed to verify their taxonomic positions. Heterogeneity was found not only within genera but also within species. *Flexibacter aggregans*, *Flexibacter aurantiacus*, *Flexibacter flexilis*, *Flexibacter roseolus*, *Flexibacter tractuosus*, and “*Microscilla sericea*” contained phylogenetically distant strains. The phylogenetic structure of each genus is discussed below.

Phylogenetic structure of *Flexibacter* and *Flexithrix*

The genus *Flexibacter* accommodates 17 approved and validated species. The genus *Flexithrix* contains only 1 species with 1 strain. These 18 species of *Flexibacter* and *Flexithrix* diverged into 20 phylogenetic groups (groups 1–8, 10–17, 19, 20, 22, and 24), that were distant from each other at the genus level or above. Groups 1–4 were located in the family *Flavobacteriaceae*, while groups 5–24, including the type species *Flexibacter flexilis* (group 11), were not. Thus, the heterogeneity of the genus exceeded the family level.

Flexibacter flexilis (group 11), the type species of the genus *Flexibacter*, was isolated from the other species. This result suggests that the genus *Flexibacter* should be restricted to *Flexibacter flexilis*. All strains in this species clustered together except for 1 non-validated subspecies, “*Flexibacter flexilis* subsp. *pelliculosus*,” that was independent from the other known taxa (group 12). The 16S rDNA similarity between “*Flexibacter flexilis* subsp. *pelliculosus*” and the

Table 2. 16S rRNA sequence similarity matrix for the

Species and strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 <i>Flexibacter aggregans</i> IFO 15975	100	92.3	90.9	91.1	91.4	91.2	92.2	91.4	91.7	92.4	88.5	82.1	82.4	81.9	81.9	81.8	86.0	82.5	82.5	82.5	82.4
2 <i>Flexibacter tractuosus</i> IFO 15980		100	91.3	91.7	91.0	91.9	92.0	92.2	92.4	92.3	90.1	82.7	83.1	83.4	83.4	82.8	85.8	82.4	82.4	82.4	82.7
3 " <i>Flexibacter aurantiacus</i> subsp. <i>copepodarum</i> " IFO 15978 ^T			100	97.8	98.1	97.0	91.8	91.1	91.3	91.4	89.3	82.6	83.0	82.6	82.6	82.4	85.1	83.5	83.5	83.5	83.8
4 <i>Tenacibaculum ovolyticum</i> (<i>Flexibacter ovolyticus</i>) IFO 15947 ^T				100	96.6	96.4	91.8	91.0	91.1	91.4	89.1	81.8	82.1	81.8	81.8	81.7	85.1	83.1	83.1	83.1	83.2
5 " <i>Flexibacter echinocida</i> " F11 (AY006470)					100	96.3	92.0	91.1	91.6	91.7	89.7	82.7	83.1	82.7	82.7	82.6	85.8	84.0	84.0	84.0	83.9
6 <i>Flexibacter maritimus</i> IFO 15946 ^T						100	92.0	91.0	91.2	91.8	89.0	82.6	83.1	82.8	82.8	82.8	85.4	83.8	83.8	83.8	83.7
7 <i>Flexibacter psychrophilus</i> IFO 15942 ^T							100	96.7	96.7	96.4	88.7	83.1	83.3	82.6	82.6	82.7	85.1	83.9	83.9	83.9	83.2
8 " <i>Flexibacter aurantiacus</i> subsp. <i>excathedrus</i> " IFO 16024 ^T								100	99.3	95.6	88.6	82.9	83.0	82.8	82.8	82.6	85.0	83.9	83.9	83.9	83.1
9 <i>Flexibacter columnaris</i> IFO 15943 ^T									100	95.3	89.0	82.8	82.9	82.7	82.7	82.7	84.9	83.8	83.8	83.8	83.0
10 <i>Flexibacter aurantiacus</i> IFO 15970 ^T										100	89.7	83.9	83.9	83.3	83.3	83.1	85.7	84.2	84.2	84.2	83.3
11 " <i>Flexibacter aggregans</i> subsp. <i>catalaticus</i> " IFO 15977 ^T											100	85.1	85.1	84.7	84.7	84.8	87.7	86.0	86.0	86.0	85.1
12 <i>Flexibacter sancti</i> IFO 16034												100	99.0	98.6	98.6	97.2	85.5	84.9	84.9	84.9	84.1
13 <i>Flexibacter filiformis</i> IFO 15056 ^T													100	98.5	98.5	97.8	86.0	84.9	84.9	84.9	83.9
14 <i>Flexibacter sancti</i> IFO 16033														100	100	97.6	86.0	84.7	84.7	84.7	84.0
15 <i>Flexibacter sancti</i> IFO 15057 ^T															100	97.6	86.0	84.7	84.7	84.7	84.0
16 <i>Flexibacter japonensis</i> IFO 16041 ^T																100	86.3	84.2	84.2	84.2	83.9
17 <i>Flexibacter canadensis</i> IFO 15130 ^T																	100	85.8	85.8	85.8	87.5
18 <i>Flexibacter roseolus</i> IFO 16486																		100	100	100	91.7
19 <i>Flexibacter elegans</i> IFO 15055 ^T																			100	100	91.7
20 <i>Flexibacter ruber</i> IFO 16675																				100	91.7
21 <i>Microscilla marina</i> IFO 16560 ^T																					100
22 <i>Flexibacter ruber</i> IFO 16677 ^T																					
23 <i>Flexibacter flexilis</i> IFO 15060 ^T																					
24 <i>Flexibacter flexilis</i> IFO 16025																					
25 <i>Flexibacter flexilis</i> IFO 16026																					
26 <i>Flexibacter flexilis</i> IFO 16027																					
27 " <i>Flexibacter flexilis</i> subsp. <i>pelliculosus</i> " IFO 16028 ^T																					
28 <i>Flexibacter litoralis</i> IFO 15988 ^T																					
29 <i>Flexibacter roseolus</i> IFO 16030																					
30 <i>Flexibacter polymorphus</i> IFO 16703 ^T																					
31 <i>Flexibacter roseolus</i> IFO 16707 ^T																					
32 " <i>Microscilla sericea</i> " IFO 15983 ^T																					
33 <i>Flexibacter tractuosus</i> IFO 15979																					
34 <i>Flexibacter tractuosus</i> IFO 15989 ^T																					
35 <i>Flexibacter tractuosus</i> IFO 15981																					
36 " <i>Microscilla furvescens</i> " IFO 15994 ^T																					
37 <i>Flexibacter tractuosus</i> IFO 16482																					
38 <i>Flexibacter tractuosus</i> IFO 16035																					
39 <i>Flexibacter aggregans</i> IFO 15974																					
40 " <i>Microscilla sericea</i> " IFO 16561																					
41 <i>Flexibacter aggregans</i> IFO 15973																					
42 <i>Flexibacter aggregans</i> IFO 15976 ^T																					
43 <i>Flexibacter aggregans</i> IFO 15990																					
44 <i>Flexithrix dorotheae</i> IFO 15987 ^T																					
45 " <i>Microscilla arenaria</i> " IFO 15982 ^T																					
46 <i>Flexibacter tractuosus</i> IFO 16037																					
47 <i>Flexibacter tractuosus</i> IFO 16038																					

^aPhylogenetic group recognized by 16S rRNA sequence analysis.

% Similarity

Group^a

22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	
83.3	82.5	82.6	82.6	82.6	81.8	82.7	82.5	83.6	82.7	84.8	84.8	85.1	85.1	84.4	83.5	83.5	85.1	85.2	84.7	84.7	84.7	84.7	81.4	84.3	83.9	1
83.3	81.9	82.0	82.1	82.1	82.3	84.6	83.0	83.6	82.7	84.4	84.4	84.7	84.6	84.3	82.9	82.9	84.0	83.4	84.2	84.2	84.2	84.2	82.3	82.8	83.1	2
83.9	81.6	81.7	81.6	81.6	81.8	83.3	82.4	84.7	81.6	84.6	84.6	84.7	84.9	84.8	84.2	84.2	83.9	82.9	83.5	83.5	83.5	83.5	83.1	84.4	84.5	3
83.7	81.2	81.3	81.2	81.2	81.4	83.2	82.0	84.4	81.4	83.9	83.9	84.1	84.3	84.7	83.8	83.8	83.4	82.5	83.3	83.3	83.3	83.3	82.9	84.2	84.2	3
84.6	82.0	82.1	82.0	82.0	82.4	83.1	82.4	84.7	82.8	85.6	85.6	85.6	85.9	85.7	84.9	84.9	84.9	84.0	84.6	84.6	84.6	84.6	83.1	84.9	84.3	3
84.1	81.8	81.8	81.8	81.8	81.7	83.3	82.6	84.8	81.4	85.1	85.1	85.3	85.6	84.2	84.0	84.0	83.9	83.6	84.3	84.3	84.3	84.3	83.4	85.1	85.1	3
82.9	82.8	82.9	82.8	82.8	82.6	83.5	83.1	85.1	83.3	84.5	84.5	84.7	85.0	85.2	83.4	83.4	84.4	84.9	84.1	84.1	84.1	84.1	81.9	83.3	82.7	4
83.4	83.3	83.4	83.5	83.5	82.7	83.3	82.9	85.5	83.5	84.6	84.6	84.7	84.4	85.1	83.6	83.6	84.9	84.4	84.3	84.3	84.3	84.3	82.6	83.5	83.0	4
83.5	83.0	83.1	83.0	83.0	82.5	83.5	82.8	85.4	83.4	84.3	84.3	84.4	84.4	85.2	83.5	83.5	84.5	84.3	83.9	83.9	83.9	83.9	82.6	83.7	83.1	4
83.9	83.1	83.2	83.2	83.2	83.4	82.7	83.0	85.4	83.4	84.2	84.2	84.2	84.0	84.8	83.4	83.4	84.7	84.5	84.2	84.2	84.2	84.2	82.8	84.2	83.6	4
86.0	84.7	84.7	84.6	84.6	84.1	83.1	84.2	85.1	84.0	85.5	85.5	85.8	85.8	86.6	86.0	86.0	86.8	85.1	86.0	86.0	86.0	86.0	84.3	84.2	84.3	5
85.2	83.7	83.8	83.6	83.6	83.1	81.6	82.6	84.1	83.4	84.7	84.7	84.3	84.1	84.7	84.6	84.6	85.2	84.0	85.4	85.4	85.4	85.4	83.1	83.2	83.1	6
85.0	83.8	83.9	83.7	83.7	83.2	82.0	82.9	84.4	83.6	85.1	85.1	84.7	84.7	85.0	84.7	84.7	85.6	84.3	85.5	85.5	85.5	85.5	83.1	83.5	83.2	6
85.2	83.9	83.9	83.9	83.9	83.0	82.1	82.3	83.9	83.0	84.9	84.9	84.5	84.1	84.7	84.9	84.9	85.7	84.2	85.4	85.4	85.4	85.4	82.9	83.5	83.3	6
85.2	83.9	83.9	83.9	83.9	83.0	82.1	82.3	83.9	83.0	84.9	84.9	84.5	84.1	84.7	84.9	84.9	85.7	84.2	85.4	85.4	85.4	85.4	82.9	83.5	83.3	6
84.5	83.0	83.1	83.1	83.1	82.6	82.1	82.2	83.9	83.3	84.4	84.4	84.0	84.1	84.3	84.5	84.5	85.3	84.1	85.1	85.1	85.1	85.1	82.9	83.4	83.1	6

authentic *Flexibacter flexilis* is below 87.1%. This low value supports that "*Flexibacter flexilis* subsp. *pelliculosus*" does not belong to the genus *Flexibacter*. The phenotypic difference of this subspecies has already been noted (Reichenbach, 1989b).

Flexibacter aggregans was assigned as "*Microscilla aggregans*" in Bergey's Manual of Systematic Bacteriology (Reichenbach, 1989b, c), because it is a marine organism. This species was found to be the phylogenetically heterogeneous, diverging into 4 groups (groups 1, 5, 20, and 22). The three authentic strains of *Flexibacter aggregans*, IFO 15973, IFO 15976^T (^T=type strain), and IFO 15990, constituted a tight cluster with *Flexithrix dorotheae* IFO 15987^T (group 22). This result strongly supported Reichenbach's report (1989a) of a close resemblance between the two species. *Flexibacter aggregans* IFO 15975 (group 1) belonged to the family *Flavobacteriaceae* and was the sister group of the genera *Zobellia*, *Muricauda*, and "*Aequorivita*." Both *Zobellia* (Barbeyron et al., 2001) and *Muricauda* (Bruns et al., 2001) are marine organisms like *Flexibacter aggregans*. *Flexibacter aggregans* IFO 15974 (group 20) was a sister group of *Flexibacter tractuosus* IFO 16035 and IFO 16482 (group 19). The 16S rDNA sequence similarity between group 19 and 20 was 94.9%. These two groups may be classified together in a new taxon. "*Flexibacter aggregans* subsp. *catalaticus*" IFO 15977^T (group 5) is located at the borderline (root position) of the family *Flavobacteriaceae*. Very recently, an emendation of the *Flavobacteriaceae* that omits *Blattabacterium* has been proposed (Bernardet et al., 2002). One distinctive character of *Flavobacteriaceae* is the presence of MK-6 (Bernardet et al., 2002; Nakagawa and Yamasato, 1993, 1996) as mentioned above. Analysis of the respiratory quinone of this strain will clarify its affiliation to the family. The highest 16S rDNA sequence similarity between "*Flexibacter aggregans* subsp. *catalaticus*" IFO 15977^T and others is 90.1%.

Flexibacter aurantiacus IFO 15970^T, *Flexibacter columnaris* IFO 15943^T and *Flexibacter psychrophilus* IFO 15942^T were located in the genus *Flavobacterium*. Bernardet et al. (1996) have proposed that *Flexibacter columnaris* and *Flexibacter psychrophilus* should be transferred to the genus *Flavobacterium* as *Flavobacterium columnare* and *Flavobacterium psychrophilum*, respectively, and that *Flexibacter aurantiacus* is the junior subjective synonym of the *Flavobacterium johnsoniae*. Two non-validated subspecies exist in *Flexibac-*

ter aurantiacus. Of these, "*Flexibacter aurantiacus* subsp. *excathedrus*" IFO 16024^T was found to belong to the genus *Flavobacterium*. However, the similarity value was sufficiently low (95.6%) to separate this strain from *Flexibacter aurantiacus* IFO 15970^T. Further investigation with other *Flavobacterium* species will be needed to establish a new species for this strain, because the genus *Flavobacterium* accommodates many species. The other subspecies, "*Flexibacter aurantiacus* subsp. *copepodarum*" IFO 15978^T (group 3), is a marine organism isolated from an off-shore crustacean (Lewin, 1969) and positioned in the genus *Tenacibaculum* (discussed below).

Flexibacter canadensis IFO 15130^T (group 7) was a sister group of the genera *Sphingobacterium* and *Pedobacter*. This relationship has been shown in our previous studies (Nakagawa and Yamasato, 1993, 1996). Steyn et al. (1998) proposed the family *Sphingobacteriaceae* to encompass the genera *Sphingobacterium*, *Pedobacter*, and *Flexibacter canadensis*. *Flexibacter canadensis* was located at the borderline of the *Sphingobacteriaceae*. This family is characterized by the presence of sphingolipids, but we could not detect a long-chain base derived from sphingolipids in *Flexibacter canadensis* (data not shown). We suggest that *Flexibacter canadensis* should be excluded from the family as a distinct genus.

Flexibacter elegans IFO 15055^T clustered with *Flexibacter roseolus* IFO 16486 and *Flexibacter ruber* IFO 16675 (group 8). These three strains showed identical 16S rDNA sequences. *Flexibacter roseolus* IFO 16486 and *Flexibacter ruber* IFO 16675 were each the type strain of the species deposited in The National Collections of Industrial and Marine Bacteria, Ltd. (Aberdeen, Scotland, UK) by R. A. Lewin. However, BCCMTM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent (Gent, Belgium) (1998) suggested that these two strains are not authentic and different from the type strains deposited in American Type Culture Collection (Manassas, VA, USA). Our analysis strongly supports this suggestion. The type strains deposited in ATCC showed different sequences: *Flexibacter roseolus* IFO 16707^T belonged to group 16, and *Flexibacter ruber* IFO 16677^T belonged to group 10.

Flexibacter filiformis IFO 15056^T, *Flexibacter japonensis* IFO 16041^T, and *Flexibacter sancti* IFO 15057^T, IFO 16033, and IFO 16034 were closely related to the genus *Chitinophaga* (group 6). The genus

Chitinophaga is characterized by its ability to degrade chitin (Skerman, 1989). Interestingly, the degradation of chitin by *Flexibacter filiformis* has been reported (Reichenbach, 1989b), although it is unknown for the remaining two *Flexibacter* species.

Flexibacter litoralis was an independent species from other organisms in the cfb phylum (group 13). Its sister groups were *Flexibacter roseolus* IFO 16030 (group 14), *Flexibacter polymorphus* IFO 16703^T (group 15), and *Flexibacter roseolus* IFO 16707^T (group 16). Each group should be classified as an independent genus, because 16S rDNA sequence similarity among these four groups was below 92.6%. Both *Flexibacter litoralis* and *Flexibacter polymorphus* fit not only the genus *Flexibacter* in their seawater requirement but also *Microscilla* in the low G+C content of their DNA (Reichenbach, 1989c). DNA:rRNA hybridization studies showed that *Flexibacter roseolus* and *Flexibacter ruber* (group 10) have not related with the *Cytophaga-Flavobacterium* complex (Bauwens and De Ley, 1981). However, it is clear that both species belonged to the cfb phylum and that each had an independent position in the phylum. *Flexibacter roseolus* was a heterogeneous species: it included 2 phylogenetically different strains (IFO 16707^T and IFO 16030) with 16S rDNA similarity of 87.8%, indicating that strain IFO 16030 does not belong to *Flexibacter roseolus*.

Flexibacter maritimus and *Flexibacter ovolyticus* have been transferred to the new genus *Tenacibaculum* (Suzuki et al., 2001) (group 3). Our study showed that two other species, "*Flexibacter aurantiacus* subsp. *copepodarum*" IFO 15978^T and "*Flexibacter echiniciida*," which is found only in the DNA database, should be classified in the genus *Tenacibaculum*. "*Flexibacter aurantiacus* subsp. *copepodarum*" is a marine organism, as is *Tenacibaculum*.

Flexibacter tractuosus is also called "*Microscilla tractuosa*," because it is a marine bacterium. Eight strains have been assigned to the species. Our phylogenetic studies revealed that this species was quite heterogeneous and diverged into 4 distinct groups (groups 2, 17, 19, 24), of which only group 2 was located in the *Flavobacteriaceae*. The type strain of the species, IFO 15989^T, clustered with IFO 15979, IFO 15981, and "*Microscilla sericea*" IFO 15983^T (group 17). This group is considered to be the genuine *Flexibacter tractuosus*, which should be excluded from *Flexibacter*. *Flexibacter tractuosus* IFO 15980 (group

2) clustered with the marine genera *Psychroflexus* and *Salegentibacter*. *Flexibacter tractuosus* IFO 16035 and IFO 16482 (group 19), and IFO 16037 and IFO 16038 (group 24) each constituted independent clusters.

Phylogenetic structure of *Microscilla*

The genus *Microscilla* includes only one approved species and three non-validated species. The five strains examined in the four species in the genus diverged into five distinct clusters (groups 9, 17, 18, 21, 23).

Microscilla marina, the type species of the genus *Microscilla*, was an isolated species (group 9). This result means that other invalid *Microscilla* species should not be included in the genus. "*Microscilla arenaria*" IFO 15982^T showed a close relationship with *Flammeovirga aprica* (group 23). The 16S rRNA sequence (accession no. M60455) of the type strain of "*Microscilla arenaria*" deposited in the DNA databank occupied a position identical to that of group 22 and was quite different from that determined in this study. The sequence of M60455 was derived from ATCC 23161^T, but this strain is no longer available because it has been contaminated. It is possible that the origin of the sequence of M60455 is not an authentic type strain of "*Microscilla arenaria*." "*Microscilla furvescens*" (group 18) is a sister group of group 17. Both groups are marine organisms and their 16S rDNA sequence similarity is 93.5%. In "*Microscilla sericea*," the two strains were phylogenetically different. The type strain (IFO 15983^T) clustered with *Flexibacter tractuosus* (group 17) and may be classified in the same taxon. The other strain, IFO 16561 occupied an independent position that seemed to require a new taxon.

Conclusion

The 16S rRNA sequence analysis revealed that the genera *Flexibacter*, *Flexithrix*, and *Microscilla* were separated into 24 phylogenetic groups that were remote from each other at genus level or higher. It is clear that the taxonomy of *Flexibacter*, *Flexithrix*, and *Microscilla* should be reorganized on the basis of the phylogenetic relationships revealed in this study. However, comprehensive taxonomic studies on these genera have not been satisfactorily performed, because few phenotypic, physiologic, and chemotaxonomic features have been reported. Accumulation of these data will lead to an improved classification of these organisms.

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