Phylogenetic diversity of the genus *Cytophaga* revealed by 16S rRNA sequencing and menaquinone analysis

YASUYOSHI NAKAGAWA* and KAZUHIDE YAMASATO

Institute of Applied Microbiology, University of Tokyo, 1-1, Yayoi 1-chome, Bunkyo-ku, Tokyo 113, Japan

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To clarify the intra- and intergeneric relationships of the genus *Cytophaga*, 16S rRNA sequences and respiratory isoprenoid quinones were determined for the type strains of the 21 validly published species and one isolate in the genus *Cytophaga*. The sequence analysis revealed extreme heterogeneity of this genus, which diverged into nine distinct lines of descent. Each lineage of *Cytophaga* was characterized by possessing either menaquinone-6 (MK-6) or MK-7. The MK-6-possessing species were located in the two lineages that were remote from MK-7 species. One of the MK-6 lineages was composed only of terrestrial species and the other only of marine species. *Flavobacterium aquatile*, the type species of the genus *Flavobacterium*, was located in the MK-6 terrestrial lineage. The terrestrial *Cytophaga* species with MK-6 should be transferred to the genus *Flavobacterium*. The marine facultative anaerobes with MK-7 were located in the bacteroides branch, and possessed signature sequences with features intermediate between the bacteroides and the flavobacteria subdivisions. *Cytophaga hutchinsonii*, the type species of the genus *Cytophaga*, had a close relationship only with *Cytophaga aurantiaca*. The genus *Cytophaga* should be restricted to these two cellulose-degrading species. The genus *Cytophaga* is so heterogeneous that it should be divided into several genera and higher taxa in accordance with the phylogenetic relationships.

Introduction

The genus *Cytophaga* was described by Winogradsky (1929) for aerobic cellulolytic soil bacteria with probable gliding motility. Stanier (1940, 1941, 1942, 1947) investigated Gram-negative bacteria that could move by gliding and decompose cellulose, agar and/or chitin, and suggested that the gliding motility and the ability to degrade biomacromolecules were indispensable characters for the concept of this genus. The generic concept given by Stanier has been accepted in principle (Leadbetter, 1974; Reichenbach, 1989a). Bergey *et al.* (1923) described the genus *Flavobacterium* for aerobic, yellow to orange pigmented rods. Its original definition was very vague, and included Gram-negative species, nonmotile or motile with flagella, aerobic or facultatively anaerobic, and even Gram-positive species. The extent of heterogeneity of the genus has been reduced progressively

until Holmes *et al.* (1984) restricted the genus *Flavobacterium* to Gram-negative aerobic, nonmotile rods with low G+C content of DNA (31–42 mol%).

Several taxonomic investigations described in the review by Shewan & McMeekin (1983) and by others (Callies & Mannheim, 1978; Christensen, 1977; Hayes, 1977; Oyaizu & Komagata, 1981) revealed unreasonable overlapping of phenotypic and chemotaxonomic characters, such as G+C contents of DNA and cellular fatty acid composition, between the genera *Flavobacterium* and *Cytophaga*. Concerning the isoprenoid quinone characters, the species of both genera were characterized by possessing menaquinone 6 (MK-6) or menaquinone 7 (MK-7) as the major quinone (Oyaizu & Komagata, 1981), and the two genera could not be differentiated. Further, the isoprenoid quinone of *Cytophaga* species has not been fully investigated. Only gliding motility could be regarded as a distinguishing feature for the two genera, but it is sometimes not easy to discern (Henrichsen, 1972; McMeekin & Shewan, 1978; Perry, 1973). Accordingly, the two genera have been called the *Flavobacterium*–*Cytophaga* complex. By DNA:rRNA hybridization study, Bauwens & De Ley (1981) clarified the intermingling of the phylogenetic locations of species of these two genera and others.

* Author for correspondence. Present address: Institute for Fermentation, Osaka, 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532, Japan. Tel. 81 6 302 7281; fax. 81 6 300 6814.

The nucleotide sequence data reported in this paper have been submitted to DDBJ and have been assigned the accession numbers D12654 to D12675.

Table 1. The phenotypic, physiologic and chemotaxonomic characters of the strains of *Cytophaga* species examined

Species and strain*		Habitat†	G + C‡ (mol %)	Relation to oxygen†	Quinone system‡	Accession number
<i>C. hutchinsonii</i>	DSM 1761	Soil	39	Strict aerobe	MK-7	D12663
<i>C. aurantiaca</i>	NCIMB 8628	Soil	42	Strict aerobe	MK-7	D12658
<i>C. diffluens</i>	NCIMB 1402	Marine	42	Strict aerobe	MK-7	D12660
<i>C. aprica</i>	ATCC 23126	Marine	35	Strict aerobe	MK-7	D12655
<i>C. arvensicola</i>	IAM 12650	Soil	46/47	Strict aerobe	MK-7	D12657
<i>C. agarovorans</i>	NCIMB 2217	Marine	41	Facultative anaerobe	MK-7	D12654
<i>C. fermentans</i>	NCIMB 2218	Marine	39	Facultative anaerobe	MK-7	D12661
<i>C. salmonicolor</i>	NCIMB 2216	Marine	37	Facultative anaerobe	MK-7	D12672
<i>C. latercula</i>	ATCC 23177	Marine	34	Strict aerobe	MK-6	D12665
<i>C. lytica</i>	ATCC 23178	Marine	33	Strict aerobe	MK-6	D12666
<i>C. marina</i>	JCM 8137	Marine	31	Strict aerobe	MK-6	D12667
<i>C. marinoflava</i>	NCIMB 397	Marine	37	Strict aerobe	MK-6	D12668
<i>C. uliginosa</i>	NCIMB 1863	Marine	42	Strict aerobe	MK-6	D12674
<i>C. aquatilis</i>	IAM 12365	Freshwater	32/34	Facultative anaerobe	MK-6	D12656
<i>C. columnaris</i>	NCIMB 2248	Freshwater	30	Strict aerobe	MK-6	D12659
<i>C. flevenensis</i>	IFO 14960	Freshwater	33/35	Strict aerobe	MK-6	D12662
<i>C. johnsonae</i>	IFO 14942	Soil, freshwater	33	Strict aerobe	MK-6	D12664
<i>C. pectinovora</i>	NCIMB 9059	Soil	34	Strict aerobe	MK-6	D12669
<i>C. psychrophila</i>	NCIMB 1947	Freshwater	32	Strict aerobe	MK-6	D12670
<i>C. saccharophila</i>	NCIMB 2072	Freshwater	32	Strict aerobe	MK-6	D12671
<i>C. succinicans</i>	IFO 14905	Freshwater	38	Facultative anaerobe	MK-6	D12673
<i>Cytophaga</i> sp.	L-43-1-N	Freshwater§	33§	ND	MK-6§	D12675

* All are type strains except for *Cytophaga* sp. L-43-1-N. Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Micro-organisms, Institute of Physical and Chemical Research (RIKEN), Wako, Japan; NCIMB, The National Collection of Industrial and Marine Bacteria Ltd, Aberdeen, UK.

† From Reichenbach (1989a). ND, Not determined.

‡ Data from the present study.

§ H. Kojima, Japan Leather Research Institute, Tokyo, Japan (personal communication).

|| Accession numbers of 16S rRNA sequences in DDBJ database.

Reichenbach (1989a) described several new species and combinations in the genus *Cytophaga* which were from both terrestrial and marine sources. However, he stated that the genus *Cytophaga* is 'a rather heterogeneous assembly of organisms which certainly do not belong to one single genus'.

Phylogenetically, the genus *Cytophaga*, with the genera *Flavobacterium*, *Bacteroides* and others, constitutes one of the eleven phyla among eubacteria (Woese, 1987) based on cataloguing (Paster *et al.*, 1985) and sequencing (Weisburg *et al.*, 1985) of 16S rRNA. In order to throw light on the intra- and intergeneric relationships for the genus *Cytophaga*, we undertook the sequencing of 16S rRNA and the determination of respiratory isoprenoid quinone systems.

Methods

Bacterial strains and cultivation. The 21 type strains in the genus *Cytophaga*, covering all the validly published species, and one isolate, were used (Table 1). *C. marina* is now recognized as a junior subjective synonym of *Flexibacter maritimus* (Holmes, 1992). The strains of *C.*

hutchinsonii and *C. aurantiaca* were grown in *Cytophaga hutchinsonii* broth (DSM Catalogue of Strains, 4th edn, p. 295, 1989) [Casitone (Difco), 3 g; CaCl₂·2H₂O, 1.36 g; yeast extract (Oriental Yeast Co.), 2 g; cellobiose, 5 g; distilled water, 1 litre; pH 7.2] and the strains of *C. aquatilis*, *C. pectinovora*, *C. saccharophila* and *Cytophaga* sp. L-43-1-N in Nutrient broth (Oxoid, no. CM1). Enriched *Cytophaga* broth (NCIMB Catalogue of Strains, p. 165, 1990) [tryptone, 2.0 g; beef extract (Kyokuto Pharmaceutical Industries), 0.5 g; yeast extract, 0.5 g; anhydrous sodium acetate, 0.2 g; distilled water, 1 litre; pH 7.2–7.4] was adapted for *C. arvensicola*, *C. columnaris*, *C. flevenensis*, *C. johnsonae*, *C. psychrophila* and *C. succinicans*. Marine *Cytophaga* species were cultured in Marine *Cytophaga* broth (NCIMB Catalogue of Strains, p. 174, 1990) (tryptone, 2.0 g; beef extract, 0.5 g; yeast extract, 0.5 g; sodium acetate, 0.2 g; aged sea water, 1 litre; pH 7.2–7.4). For *C. agarovorans*, *C. fermentans* and *C. salmonicolor*, SP5 liquid medium (Reichenbach, 1992) (Casitone, 9 g; yeast extract, 1 g; aged sea water, 1 litre; pH 7.2) was used. The temperature for cultivation was 27 °C except for *C. columnaris* and *C. psychrophila*, which were grown at 20 °C and 17 °C, respectively. Cells were harvested by centrifugation at the mid-exponential phase of growth.

Extraction, purification and determination of isoprenoid quinones. The procedures for extraction and purification of isoprenoid quinones were as described by Yamada & Kuraishi (1982). Menaquinone homologues were determined by using a high-performance liquid chromatograph (Millipore, model 510) equipped with a CrestPak C 18S column (4.6 × 150 mm) (Japan Spectrophotometric Co.) and a Lambda-Max

model 481 LC spectrophotometer (Millipore) at 270 nm. Samples were eluted with methanol/2-propanol (2:1, v/v) at a flow rate of 1 ml min⁻¹.

Preparation and sequencing of 16S rRNA. Total RNAs were extracted by the procedure described by Yamada & Kawasaki (1989) except for lysis of cells. Frozen cells (approximately 0.1 g, wet wt) were suspended in 1 ml TMK buffer and lysed by adding 150 µl 10% (w/v) sodium dodecylsulphate. The dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) with reverse transcriptase (Takara Shuzo Co.) (Lane *et al.*, 1985) was used for the determination of 16S rRNA sequences. The set of oligonucleotide primers employed was 350R, 5'-CTGCTGCCTCCCGTA-3'; 520R, 5'-GTATTACCGCGGCTGCTG-3'; 700R, 5'-TCTACGCATTTACCGCTAC-3'; 800R, 5'-CTAC-CAGGGTATCTAAT-3'; 920R, 5'-CCGTCAATTCATTTGAGTTT-3'; 1100R, 5'-AGGGTTGCGCTCGTTG-3'; 1240R, 5'-CATTGTAGCAGTGTGTA-3' and 1400R, 5'-CGGTGTGTACAAGCCC-3' (Takara Shuzo Co.).

Analysis of 16S rRNA sequences. The sequences were aligned against *Cytophaga hutchinsonii* DSM 1761, the type strain of the type species of the genus *Cytophaga*. The eight published sequences of *Cytophaga heparina* (EMBL accession number, M11657), *Flavobacterium aquatile* (GenBank, M28230), *Flavobacterium ferrugineum* (GenBank, M28237), *Flexibacter aurantiacus* (GenBank, M28054), *Flexibacter canadensis* (GenBank, M28055), *Flexibacter flexilis* (GenBank, M28056), *Flexibacter sancti* (GenBank, M28057) and *Bacteroides fragilis* (EMBL, M11656) (Weisburg *et al.*, 1985; Woese *et al.*, 1990b) were included for comparison. *Escherichia coli* (EMBL, V00348) (Brosius *et al.*, 1978) was included as an outgroup. A software package, TREE Version 0.1 (kindly provided by Dr N. Saitou, National Institute of Genetics, Mishima, Japan) was used to generate the homology values and the evolutionary distances (K_{nuc} values) (Kimura, 1980) and to reconstruct the phylogenetic tree based on the neighbour-joining method of Saitou & Nei (1987) from the K_{nuc} values.

Results and Discussion

Construction of a phylogenetic tree based on 16S rRNA sequence analysis

The sequencing strategy generated a continuous stretch of sequence ranging from position 110 to 1375 on the *E. coli* numbering system for each strain. The sequence data have been deposited with DDBJ and will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession numbers D12654 to D12675 (Table 1). The homology values and the K_{nuc} values are shown in Table 2. The variable regions in which insertions and/or deletions occurred which might make alignment incorrect (from positions 143 to 220, 447 to 487, 830 to 856, 991 to 1045) were excluded from the calculation in order to obtain more reliable phylogenetic relationships at higher ranks. The homology values ranged from 80.4 to 99.3% among the strains of the *Flavobacterium*-*Cytophaga* complex. This wide range and the significantly low homology values at a level of 80% observed between many pairs of strains demonstrated the phylogenetic heterogeneity of the genus *Cytophaga* (Table 2).

The phylogenetic tree shows that the genus *Cytophaga* consists of nine distinct lines of descent (Fig. 1).

C. hutchinsonii, the type species of the genus, showed a close relationship only with *C. aurantiaca*, at a homology value of 98.9%. Homology values between each of these two species and the other *Cytophaga* species were below 87.5%, which means that the other *Cytophaga* species are remote from the type species of the genus *Cytophaga*. *C. hutchinsonii* and *C. aurantiaca* share the same phenotypic characters such as degradation of cellulose, possessing MK-7 and inhabiting terrestrial environments (Table 1). Their DNA G+C contents are 39 and 42 mol% respectively (Table 1). From the above stated facts, the genus *Cytophaga* should be restricted to *C. hutchinsonii* and *C. aurantiaca*. The isoprenoid quinone specific to the genus *Cytophaga* should be regarded as MK-7. Reichenbach (1989a) stated his view that the genus *Cytophaga* should be restricted to these two species, attributing the distinguishing feature to cellulose-degrading ability.

Flexibacter flexilis, the type species of the genus *Flexibacter*, was independent of other species of *Flexibacter* and the species of *Cytophaga* and *Flavobacterium*. Woese *et al.* (1990a, b) showed that *Flexibacter flexilis* was associated with the ring-forming species (*Spirosomaceae*), and that this group was monophyletic in the bacteroides-flavobacteria phylum.

Each of the marine aerobes, *C. diffuens* and *C. aprica*, constituted a distinct lineage among the MK-7 lineages.

C. heparina and *Flexibacter canadensis* were located in the same lineage, as was reported by Woese *et al.* (1990b), and the homology value of sequences between them was 91.7%. The genus *Sphingobacterium* was separated from the species of the genus *Flavobacterium* on the basis of fatty acid composition and the typical presence of sphingolipids (Yabuuchi *et al.*, 1983). Recently, Steyn *et al.* (1992) demonstrated by DNA:rRNA hybridization that *C. heparina* was phylogenetically related to species of the genus *Sphingobacterium* and *Flexibacter canadensis*, and that the genus *Sphingobacterium* had an independent position in the *Flavobacterium*-*Cytophaga* complex. Further research is needed to clarify the precise relationship of *Sphingobacterium* to *C. heparina* and *Flexibacter canadensis*.

C. arvensicola clustered with *Flavobacterium ferrugineum* and *Flexibacter sancti* with homology values of 93.5% and 97.7%, respectively. The DNA G+C contents of these three species are characteristically higher than the others, ranging from 42.8 to 48.6 mol% (Holmes *et al.*, 1984; Oyaizu *et al.*, 1982; Reichenbach, 1989b). These three species could constitute a distinct genus. The species of the genus *Cytophaga* mentioned above are characterized by possessing MK-7 and a strictly respiratory type of metabolism.

C. agarovorans, *C. fermentans* and *C. salmonicolor* branched from the bacteroides lineage (Fig. 1). Hom-

Table 2. Homology and K_{nuc} values for sequences of 16S rRNA from *Cytophaga* species and related organisms

The values on the upper right are percentages of homology values, and the values on the lower left are K_{nuc} values. Abbreviations: B., *Bacteroides*; C., *Cytophaga*; E., *Escherichia*; F., *Flavobacterium*; Fx., *Flexibacter*.

1 <i>C. hutchinsonii</i>		98.9	87.9	87.2	85.1	88.2	87.5	84.6	84.6	83.1	83.1	85.6	85.7	85.4	83.6
2 <i>C. aurantiaca</i>	0.011		87.5	86.7	84.6	87.6	87.4	84.5	84.5	83.2	83.4	85.3	85.6	85.3	82.9
3 <i>Fx. flexilis</i>	0.131	0.137		87.1	84.0	86.3	85.8	83.3	83.5	81.4	82.4	85.7	86.6	86.1	82.2
4 <i>C. diffluens</i>	0.141	0.146	0.142		87.1	87.2	86.6	85.6	85.6	84.5	85.4	88.3	87.6	86.8	83.4
5 <i>C. aprica</i>	0.167	0.172	0.181	0.142		83.9	85.0	82.1	82.1	80.8	81.9	84.8	84.5	83.9	81.6
6 <i>Fx. canadensis</i>	0.129	0.135	0.152	0.141	0.182		91.7	86.8	86.2	84.1	84.8	88.4	88.2	87.6	84.3
7 <i>C. heparina</i>	0.137	0.138	0.157	0.148	0.168	0.088		85.1	85.2	83.7	84.6	87.4	87.4	86.9	84.6
8 <i>C. arvensicola</i>	0.173	0.174	0.189	0.160	0.206	0.145	0.167		97.7	93.5	82.5	85.8	85.1	84.7	82.6
9 <i>Fx. sancti</i>	0.173	0.174	0.186	0.160	0.206	0.153	0.165	0.024		93.1	82.9	85.8	85.1	84.5	82.7
10 <i>F. ferrugineum</i>	0.193	0.191	0.215	0.175	0.224	0.180	0.184	0.069	0.072		81.7	84.4	83.2	82.6	84.2
11 <i>B. fragilis</i>	0.193	0.189	0.201	0.163	0.209	0.170	0.173	0.200	0.196	0.212		87.5	86.9	86.4	82.7
12 <i>C. fermentans</i>	0.160	0.164	0.159	0.129	0.170	0.127	0.138	0.158	0.157	0.176	0.138		92.4	91.7	88.8
13 <i>C. salmonicolor</i>	0.159	0.160	0.148	0.137	0.174	0.129	0.138	0.168	0.168	0.192	0.144	0.081		98.7	85.1
14 <i>C. agarovorans</i>	0.163	0.164	0.155	0.146	0.182	0.136	0.143	0.172	0.175	0.200	0.151	0.088	0.013		85.5
15 <i>F. aquatilis</i>	0.186	0.196	0.204	0.189	0.212	0.177	0.173	0.198	0.197	0.179	0.199	0.121	0.167	0.162	
16 <i>C. saccharophila</i>	0.186	0.194	0.203	0.183	0.210	0.173	0.171	0.198	0.199	0.182	0.197	0.121	0.163	0.157	0.025
17 <i>C. aquatilis</i>	0.188	0.194	0.206	0.188	0.207	0.176	0.177	0.206	0.204	0.187	0.200	0.121	0.166	0.157	0.027
18 <i>C. succinicans</i>	0.187	0.198	0.207	0.190	0.208	0.176	0.178	0.201	0.203	0.189	0.202	0.129	0.169	0.163	0.027
19 <i>C. pectinovora</i>	0.193	0.196	0.213	0.192	0.208	0.181	0.177	0.206	0.206	0.189	0.201	0.129	0.169	0.163	0.036
20 <i>Cytophaga</i> sp. L-43-1-N	0.187	0.193	0.204	0.189	0.205	0.174	0.177	0.206	0.206	0.193	0.208	0.126	0.167	0.161	0.036
21 <i>C. psychrophila</i>	0.175	0.174	0.201	0.186	0.204	0.177	0.176	0.190	0.199	0.177	0.194	0.119	0.164	0.160	0.036
22 <i>C. flevensis</i>	0.190	0.192	0.200	0.183	0.205	0.174	0.175	0.190	0.190	0.176	0.191	0.121	0.162	0.159	0.042
23 <i>C. columnaris</i>	0.197	0.198	0.207	0.200	0.206	0.185	0.177	0.201	0.206	0.187	0.198	0.125	0.174	0.167	0.046
24 <i>Fx. aurantiacus</i>	0.178	0.188	0.200	0.180	0.194	0.167	0.170	0.193	0.191	0.183	0.196	0.128	0.159	0.153	0.047
25 <i>C. johnsonae</i>	0.177	0.184	0.195	0.181	0.190	0.167	0.170	0.194	0.193	0.185	0.199	0.128	0.158	0.152	0.053
26 <i>C. lytica</i>	0.199	0.200	0.208	0.174	0.183	0.159	0.161	0.204	0.213	0.196	0.192	0.128	0.163	0.164	0.082
27 <i>C. marinoflava</i>	0.197	0.206	0.206	0.172	0.189	0.156	0.164	0.203	0.212	0.196	0.192	0.129	0.162	0.163	0.083
28 <i>C. latercula</i>	0.193	0.198	0.213	0.191	0.200	0.163	0.179	0.196	0.198	0.195	0.197	0.129	0.162	0.157	0.092
29 <i>C. uliginosa</i>	0.206	0.209	0.200	0.174	0.205	0.166	0.165	0.199	0.203	0.197	0.184	0.141	0.169	0.164	0.096
30 <i>C. marina</i>	0.194	0.206	0.228	0.172	0.186	0.177	0.180	0.202	0.208	0.195	0.216	0.152	0.189	0.186	0.100
31 <i>E. coli</i>	0.240	0.239	0.267	0.232	0.250	0.243	0.249	0.262	0.254	0.260	0.276	0.257	0.261	0.269	0.271
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

ology values among them were relatively high, ranging from 91.7 to 98.7%. They were divided into two lines of descent: *C. agarovorans* and *C. salmonicolor*, and *C. fermentans*. The former constituted a tight cluster whose homology value was 98.7%. *C. agarovorans* was classified as a variety of *C. salmonicolor*, *C. salmonicolor* var. *agarovorans*, by Veldkamp (1961). It is particularly noteworthy that these three species had the same characters of possessing MK-7, being facultative anaerobes and inhabiting marine environments (Table 1). The fact that the organisms possessing respiratory and fermentative energy metabolisms took the 'intermediate' position between the aerobes and the anaerobes would imply that aerobes evolved to acquire an anaerobic metabolism in this phylogenetic group, which includes anaerobe and facultative anaerobe branches. The phylogenetically intermediate character was also manifested in the signature sequences (see below).

Correlation of the phylogenetic tree with menaquinone analysis

Woese (1987) divided the phylum into two subdivisions,

the bacteroides and the flavobacteria subdivisions, based on the topology drawn by cataloguing of 16S rRNA, and reported the presence of signature sequences which were characteristic to each subdivision. In the present results, *B. fragilis* was not topologically segregated or markedly more distant than other lineages (Table 2, Fig. 1). Contrary to the rather radiating topology of the lines of descent, the signature sequences generally hold. In the case of the three marine facultative anaerobes with MK-7, the signature sequences seem to reflect their phylogenetic positions. They had nucleotides identical to that of the bacteroides subdivision [G at position 306, A at position 947, (*C. agarovorans* was not determined) and U at position 1234]. Also the identical signatures were found in *C. fermentans* (G at position 1202) and in both *C. salmonicolor* and *C. agarovorans* (C at position 1321). Contrary to the above signatures, they shared signatures with the flavobacterium subdivision [C at position 316, G at 337, G at 1089, U at 1202 (*C. agarovorans* and *C. salmonicolor*) and U at 1321 (*C. fermentans*)]. In a sense, the MK-7-possessing, marine facultative anaerobes had features intermediate between the bacteroides and the flavobacteria subdivisions. Phylogenetic and phenotypic characters suggest that *C. agarovorans*, *C. fermentans*

1	83.6	83.4	83.5	83.1	83.5	84.4	83.3	82.7	84.2	84.3	82.6	82.7	83.1	82.1	83.0	79.5
2	83.0	83.0	82.6	82.9	83.1	84.5	83.1	82.6	83.4	83.7	82.5	82.1	82.6	81.9	82.1	79.5
3	82.3	82.1	82.0	81.5	82.2	82.4	82.5	82.0	82.5	82.9	82.0	82.1	81.5	82.5	80.4	77.5
4	83.9	83.5	83.3	83.2	83.4	83.6	83.9	82.5	84.1	84.0	84.5	84.7	83.2	84.5	84.7	80.1
5	81.9	82.1	82.0	82.0	82.2	82.3	82.2	82.1	83.0	83.3	83.9	83.4	82.5	82.2	83.6	78.8
6	84.6	84.4	84.4	84.0	84.5	84.3	84.5	83.6	85.1	85.1	85.7	86.0	85.4	85.2	84.3	79.3
7	84.7	84.3	84.2	84.3	84.3	84.4	84.4	84.3	84.8	84.8	85.6	85.4	84.1	85.2	84.1	78.9
8	82.6	82.1	82.4	82.1	82.1	83.3	83.3	82.4	83.1	83.0	82.2	82.3	82.9	82.6	82.4	78.0
9	82.6	82.2	82.3	82.1	82.1	82.6	83.3	81.9	83.2	83.1	81.5	81.6	82.6	82.3	82.0	78.5
10	84.0	83.5	83.4	83.4	83.1	84.3	84.4	83.5	83.9	83.7	82.9	82.9	82.9	82.9	83.0	78.1
11	82.9	82.6	82.5	82.5	82.1	83.1	83.3	82.7	83.0	82.7	83.2	83.2	82.7	83.7	81.4	77.0
12	88.8	88.8	88.2	88.2	88.4	89.0	88.8	88.5	88.3	88.3	88.3	88.2	88.2	87.2	86.3	78.3
13	85.4	85.2	85.0	85.0	85.1	85.3	85.5	84.5	85.7	85.8	85.4	85.5	85.5	85.0	83.4	78.0
14	85.8	85.8	85.4	85.4	85.5	85.6	85.7	85.1	86.2	86.3	85.3	85.4	85.8	85.4	83.6	77.4
15	97.6	97.3	97.3	96.5	96.5	96.5	95.9	95.6	95.5	94.9	92.3	92.1	91.4	91.0	90.7	77.3
16		98.9	98.5	98.7	98.9	96.9	97.3	94.8	97.7	97.1	92.0	91.9	91.3	90.9	90.4	77.9
17	0.011		98.5	98.0	98.5	97.3	96.9	95.1	97.1	96.9	92.5	92.0	91.3	90.6	90.6	77.5
18	0.016	0.016		97.8	98.0	96.7	97.1	95.0	97.0	96.5	92.0	91.7	91.5	90.3	90.4	77.4
19	0.013	0.020	0.023		98.7	96.5	96.9	94.2	96.9	96.7	91.8	91.2	91.0	90.4	89.7	77.3
20	0.011	0.016	0.020	0.013		96.5	96.9	94.2	97.8	97.6	92.1	91.6	90.8	89.9	89.7	77.1
21	0.032	0.027	0.034	0.036	0.036		95.9	95.8	95.6	95.6	92.1	91.6	90.9	89.7	90.3	77.4
22	0.027	0.032	0.029	0.032	0.032	0.042		94.8	96.8	96.6	91.2	90.7	90.5	90.2	89.8	77.3
23	0.054	0.050	0.052	0.060	0.060	0.043	0.054		94.2	93.9	90.8	90.5	90.7	88.9	89.2	76.9
24	0.024	0.029	0.031	0.032	0.022	0.046	0.033	0.060		99.3	91.8	91.9	90.3	89.8	90.2	77.2
25	0.029	0.032	0.036	0.034	0.025	0.046	0.035	0.063	0.007		91.8	91.6	90.2	89.3	89.9	76.8
26	0.085	0.080	0.085	0.087	0.084	0.083	0.095	0.098	0.087	0.087		98.9	93.8	92.9	91.4	77.0
27	0.086	0.085	0.089	0.095	0.090	0.089	0.100	0.102	0.086	0.090	0.011		93.8	93.1	91.5	77.0
28	0.093	0.093	0.091	0.096	0.098	0.097	0.102	0.099	0.105	0.106	0.065	0.065		89.8	90.4	77.4
29	0.098	0.101	0.105	0.104	0.109	0.111	0.106	0.120	0.111	0.117	0.075	0.072	0.110		90.4	77.0
30	0.103	0.101	0.103	0.111	0.111	0.105	0.110	0.117	0.106	0.109	0.092	0.091	0.103	0.104		77.5
31	0.263	0.268	0.269	0.271	0.274	0.269	0.271	0.277	0.272	0.279	0.277	0.277	0.270	0.276	0.269	
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31

and *C. salmonicolor* are intermediate representatives of the two subdivisions in this phylum.

All the species with MK-6 constituted a distinct cluster and were phylogenetically distant from all species with MK-7. The phylogenetic tree shows that the MK-6 cluster was further divided into two lines of descent (Fig. 1). Each of the lineages correspond completely with the ecological niche of its members. One of the lineages was composed only of terrestrial species, and the other only of marine species. The terrestrial MK-6 lineage constituted a tight cluster; homology values of 16S rRNA sequences among the members were higher than 93.9%. It contained both aerobic and facultatively anaerobic species. The latter species, *C. aquatilis* and *C. succinicans*, were closely related to each other, with a homology value of 98.5% (Table 1, Fig. 1). This terrestrial MK-6 cluster included *Flavobacterium aquatile*, the type species of the genus *Flavobacterium* (Fig. 1). Oyaizu & Komagata (1981) reported *F. aquatile* to possess MK-6. The present type strain of *F. aquatile* was that proposed as neotype strain by Weeks (1955). It was reported to be isolated from the water of the same well from which the lost type strain was isolated. The present type strain is the sole

extant strain of the species and has been reported to have gliding motility (Perry, 1973). The problems of the acceptability of the present type strain and *F. aquatile* as the type species have been discussed (Holmes & Owen, 1979; McMeekin & Shewan, 1978). Recently, the ICSB Subcommittee on the Taxonomy of *Flavobacterium*- and *Cytophaga*-like Bacteria (1991) accepted that 'the genus *Flavobacterium* would ultimately be restricted to the single strain representing the *F. aquatile* and to a few other closely related species', and 'most of the species currently placed in the genus *Flavobacterium* would ultimately be placed in several other genera'. The phylogenetic cluster which is composed of the type strain of *F. aquatile*, and several aerobic or facultatively anaerobic, MK-6-potential species of *Cytophaga* and *Flexibacter aurantiacus*, can be phenotypically delineated by possession of MK-6, terrestrial habitat and gliding motility. Their G+C contents of DNA are 30 to 38 mol% (Table 1), which is an acceptable range within a single genus. The species in the terrestrial MK-6-potential lineage should constitute a single genus; the *Cytophaga* species and *Flexibacter aurantiacus* should be transferred to the genus *Flavobacterium*. Other species

Fig. 1. The phylogenetic tree for *Cytophaga* species and related organisms based upon 16S rRNA sequence comparison. For abbreviations see Table 2.

exist in the genus *Flavobacterium*, and should be studied in the future. The marine species share the characters of strictly respiratory energy metabolism, quinone system, similar values of DNA G+C content and gliding motility, but constitute a phylogenetically rather diverse assemblage remote from the terrestrial cluster. They can not be included in either of the genera *Cytophaga* or *Flavobacterium*, because they are remote from the type species *C. hutchinsonii* and *F. aquatile*. Further phenotypic researches are needed for assigning them to a new taxon or new taxa.

Conclusions

In conclusion, the phylogenetic study revealed that the species of the genera *Cytophaga*, *Flavobacterium* and *Flexibacter* are scattered across the whole region of the bacteroides-flavobacteria phylum irrespective of the genus they were assigned to. The genera *Cytophaga*, *Flavobacterium* and *Flexibacter* should be reorganized and redefined phenotypically and chemotaxonomically based on the phylogenetic relationships revealed.

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