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# Phylogenetic analysis and taxonomic study of marine *Cytophaga*-like bacteria: proposal for *Tenacibaculum* gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov.

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**Bacterial strains were isolated from sponge and green algae which were collected on the coast of Japan and Palau. The phylogenetic relationships of these isolates among marine species of the *Cytophaga–Flavobacterium–Bacteroides* complex were analysed by using their *gyrB* nucleotide sequences and translated peptide sequences (GyrB) in addition to 16S rDNA sequences. These isolates were closely related to the previously characterized marine *Flexibacter* species, [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus*. These *Flexibacter* species are distantly related to *Flexibacter flexilis*, the type species of the genus *Flexibacter*, and phylogenetically belong to the family *Flavobacteriaceae* (according to analysis using both 16S rDNA and GyrB sequences). Their phylogenetic, chemotaxonomic and phenotypic characteristics prompted the proposal that these two species should be transferred to the new genus *Tenacibaculum*, as *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum*, respectively. Two additional new species of the genus *Tenacibaculum*, *Tenacibaculum mesophilum* gen. nov., sp. nov. (= MBIC 1140$^T$ = IFO 16307$^T$) and *Tenacibaculum amylolyticum* gen. nov., sp. nov. (= MBIC 4355$^T$ = IFO 16310$^T$), which were isolated from sponges and macroalgae, are also reported. For taxonomic considerations at the species level, the resolution of *gyrB* sequences was superior to that of 16S rDNA sequences, and the grouping based on the *gyrB* phylogram was consistent with DNA–DNA hybridization results.**

**Keywords:** *Cytophaga–Flavobacterium–Bacteroides* complex, *gyrB*, 16S rDNA, taxonomy, *Tenacibaculum* gen. nov.

## INTRODUCTION

Ecological studies have demonstrated that members of the *Cytophaga–Flavobacterium–Bacteroides* (CFB) complex constitute one of the dominant bacterial groups in the marine environment (Bowman *et al.*, 1997b; Pinhassi *et al.*, 1997). They are known as decomposers of biomacromolecules such as cellulose, agar and chitin. These abilities indicate that the CFB group plays an important role in carbon cycling in the marine environment. Some of these bacteria have been also reported as fish pathogens (Bernardet, 1998), algicidal and/or algal-lytic (Maeda *et al.*, 1998), algal-attached (Shiba & Taga, 1980; Bolinches *et al.*, 1988)

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**Abbreviation:** CFB, *Cytophaga–Flavobacterium–Bacteroides*.

and inducers of morphogenesis in macro-algae (Hanzawa *et al.*, 1993; Nakanishi *et al.*, 1996). These positive and negative interactions between bacteria and algae or other marine organisms are doubtless important to the marine ecosystem and also to the marine-product industry.

The taxonomy of the CFB complex is confused. In recent years, reclassifications and emended descriptions have been applied to these bacteria (Shah & Collins, 1988; Nakagawa & Yamasato, 1993; Bernardet *et al.*, 1996). However, the taxonomic identity of marine CFB species has not yet been resolved.

The similarity of small-subunit rRNA (16S rRNA) sequences is used as a powerful tool for bacterial classification (Olsen & Woese, 1993). However, resolution of 16S rRNA sequence analysis is insufficient to distinguish closely related organisms (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994; Clayton *et al.*, 1995). On the other hand, protein-encoding genes evolve faster than rRNA genes (rDNA), so a phylogenetic analysis based on these genes has a higher resolution than one based on the 16S rRNA sequence (Ochman & Wilson, 1987; Yamamoto & Harayama, 1996). Yamamoto & Harayama (1995) have developed a PCR amplification and direct sequencing system for the DNA gyrase B subunit gene (*gyrB*) and have shown that a phylogenetic analysis based on the *gyrB* sequence has a greater degree of resolution than that based on the 16S rRNA sequence (Yamamoto & Harayama, 1996).

We have isolated bacterial strains of the CFB complex from sponge samples collected from the coastal environment of Japan and Palau. In the present study, five of these bacterial isolates and two similar strains that had been isolated from the surfaces of macro-algae were selected for phylogenetic and taxonomic studies. Of these strains, five were thought to belong to new bacterial taxa that are phylogenetically related to the fish-pathogenic species [*Flexibacter*] *ovolyticus* (Hansen *et al.*, 1992) and [*Flexibacter*] *maritimus* (Wakabayashi *et al.*, 1986) (square brackets indicate generically misclassified bacteria). Because these four bacterial species were only distantly related to *Flexibacter flexilis*, the type species of the genus *Flexibacter*, we propose the reclassification of [*Flexibacter*] *ovolyticus* and [*Flexibacter*] *maritimus* into *Tenacibaculum* gen. nov., as *Tenacibaculum ovolyticum* comb. nov. and *Tenacibaculum maritimum* comb. nov., as well as the description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov.

## METHODS

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. Strains MBIC 1140$^T$, MBIC 1543 and MBIC 1544 were isolated from homogenates of sponge samples that had been collected from the coast of Japan, strains MBIC 4356 and MBIC 1357 were isolated from homogenates of sponge samples that had been collected in Palau, while strains MBIC 4355$^T$ and MBIC 4357 were isolated from the surfaces of marine macro-algae sampled from the coast of Japan. A 1/5 LBM medium [2·0 g tryptone and 1·0 g yeast extract in 1000 ml Jamarin S synthetic sea water (Jamarin Laboratory) at pH 7·2] was used for cultivating these isolates. The media used for the type and reference strains are listed in Table 1. The strains of [*Flexibacter*] *ovolyticus* and *Polaribacter glomeratus* were incubated at 15 °C, whereas the other strains were incubated at 25 °C.

**Phenotypic characterization.** Tests on the utilization of several carbon sources employed a basal medium containing 0·2 g $NaNO_3$, 0·2 g $NH_4Cl$ and 0·05 g yeast extract in 1000 ml Jamarin S synthetic sea water at pH 7·2. Each carbon source was added to the basal medium at a final concentration of 0·4 % (w/v). The growth of the strains on each substrate was recorded by measuring the optical density at 660 nm, up to the 21st day, with a spectrophotometer. The ability of the strains to degrade macromolecules was investigated according to Smibert & Krieg (1981) and Lewin & Lounsbery (1969) with a medium which had been prepared from Jamarin S synthetic sea water. The production of nitrate reductase, oxidase and catalase by the strains was also investigated according to Smibert & Krieg (1981). The ability of the strains to grow under anaerobic conditions and by nitrate respiration was tested with the Oxoid anaerobic system. To test their growth at different temperatures, our isolates were incubated on 1/5 LBM agar plates at 6, 15, 20, 30, 37, 40 and 45 °C up to the 21st day. To test their growth response to salinity, a 1/5 LBM broth containing 0, 10, 30, 50, 70 or 100 % (v/v) strengths of Jamarin S synthetic sea water or one containing 1, 3, 5, 7 or 10 % (w/v) NaCl was used. Growth at different pH values was checked on 1/5 LBM broth at pH values in the range 3–11. The morphological characteristics of our isolates were checked with an Axiophot 2 optical microscope (Zeiss), using cells cultured at 20 °C on 1/5 LBM agar plates. Gliding motility was observed, as described by Perry (1973) with some modifications. Cells in 1/5 LBM liquid cultures were spread on both agar-coated and -uncoated coverslips, and the cell movement on the surfaces was examined at × 1000 magnification.

**Chemotaxonomic analysis.** Isoprenoid quinones were extracted and analysed according to the method of Nakagawa & Yamasato (1993). Pigments were extracted from cells cultured for 18 h, by exposure to acetone for a few minutes. After centrifugation, the supernatant was immediately injected into an HPLC system equipped with a reversed-phase TSK gel ODS-80Ts column (length 15 cm, diameter 4·6 mm, 5 μm particle size; Tosoh). The pigments were eluted with methanol : water (9 : 1). The initial flow rate of 1 ml $min^{-1}$ was changed to 2 ml $min^{-1}$ 9 min after the sample had been injected. The eluted pigments were detected with a UV-visible device (UV8010; Tosoh) at 440 nm or by a photodiode array detector (SPD-M6A; Shimadzu) and then identified by comparing the retention times and absorption spectra with those of authentic standards. Flexirubin pigment was detected by the bathochromic shift test using a 20 % (w/v) KOH solution (Fautz & Reichenbach, 1980).

**Genotypic analysis.** The method for extracting and purifying genomic DNA has been described previously (Suzuki *et al.*, 1999). The extracted DNA was further purified with 2 g hydroxyapatite powder (DNA-grade Bio-Gel HTP; Bio-

***Table 1.*** Strains used in this study

Strains in bold were used for phenotypic studies. Sequences in parentheses were from GenBank. Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IAM, IAM Culture Collection Center for Cellular and Molecular Research, University of Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; MBIC, Collection of the Marine Biotechnology Institute, Kamaishi, Japan; NCIMB, National Collection of Industrial Food and Marine Bacteria, Aberdeen, UK.

| Species/strain designation | Strain no. | Isolation source | Medium | Accession nos: | |
|---|---|---|---|---|---|
| | | | | *gyrB* | 16S rDNA |
| Isolates: | | | | | |
| KK10306 | **MBIC 1140$^T$** | Sponge, Japan | 1/5 LBM | AB032585 | AB032501 |
| DK30213 | **MBIC 1543** | Sponge, Japan | 1/5 LBM | AB032589 | AB032502 |
| C808 | **MBIC 4356** | Sponge, Palau | 1/5 LBM | AB032587 | AB032503 |
| C872 | **MBIC 4357** | Green algae, Palau | 1/5 LBM | AB032588 | AB032504 |
| C526 | **MBIC 4355$^T$** | Green algae, Palau | 1/5 LBM | AB032586 | AB032505 |
| PJSA11 | MBIC 1357 | Sponge, Palau | 1/5 LBM | AB034212 | AB032514 |
| DK30223 | MBIC 1544 | Sponge, Japan | 1/5 LBM | AB034218 | AB032509 |
| Reference strains: | | | | | |
| *Bacteroides vulgatus* | IFO 14291$^T$ | Human faeces | | (AB15029) | |
| *Capnocytophaga canimorsus* | ATCC 35979$^T$ | Human blood after dog bite | ATCC medium 434 | AB034211 | (L14637) |
| *Capnocytophaga gingivalis* | ATCC 33624$^T$ | Periodontal lesion | DSM medium 340 | AB032576 | (X67608) |
| *Capnocytophaga ochracea* | ATCC 27872$^T$ | Human oral cavity | DSM medium 340 | AB032577 | |
| *Cellulophaga lytica* | IAM 14306$^T$ | Beach mud, Costa Rica | 1/5 LBM | AB034216 | (D12666) |
| *Cellulophaga lytica* | IFO 15986 | Sea-water aquarium outflow, CA, USA | 1/5 LBM | AB034217 | AB032510 |
| *Cellulophaga lytica* | IFO 16020 | Beach silt, Mexico | 1/5 LBM | AB034213 | AB032511 |
| *Cellulophaga lytica* | IFO 16021 | Sea water, Canada | 1/5 LBM | AB034214 | AB032512 |
| *Cellulophaga lytica* | IFO 16022 | Sea-water aquarium outflow, CA, USA | 1/5 LBM | AB034215 | AB032513 |
| [*Cytophaga*] *fermentans* | IFO 15936$^T$ | Marine mud | | (AB15031) | |
| *Cytophaga hutchinsonii* | IAM 12607$^T$ | Soil | | (AB15037) | (M58768) |
| [*Cytophaga*] *latercula* | **IAM 14305$^T$** | Sea-water aquarium outflow, USA | 1/5 LBM | AB034223 | (D12665) |
| [*Cytophaga*] *marinoflava* | **IFO 14170$^T$** | Sea water, offshore, Scotland, UK | 1/5 LBM | AB034221 | '(M58770), (D12668)' |
| [*Cytophaga*] *uliginosa* | **ATCC 14397$^T$** | Marine sediment | 1/5 LBM | AB034224 | (D12674) |
| *Flammeovirga aprica* | ATCC 23126$^T$ | Rocky sand, HI, USA | 1/5 LBM | AB034220 | |
| *Flavobacterium aquatile* | IAM 12316$^T$ | Deep well, UK | IAM B-9 medium | AB034225 | (M62797) |
| *Flavobacterium johnsoniae* | IAM 14304$^T$ | Soil, UK | IFO medium 275 | AB034222 | (M59051) |
| *Flectobacillus major* | NCIMB 11363$^T$ | Algal culture | NCIMB medium 81 | AB034234 | |
| [*Flavobacterium*] *salegens* | DSM 5424$^T$ | Organic lake, Antarctica | 1/5 LBM | AB034227 | (M92279) |
| [*Flexibacter*] *canadensis* | IFO15130$^T$ | Soil, Canada | IFO medium 278 | AB032584 | |
| [*Flexibacter*] *elegans* | IFO 15055$^T$ | Hot spring, New Zealand | IFO medium 204 | AB032580 | (M58783) |
| *Flexibacter flexilis* | IFO 15060$^T$ | Pond, Costa Rica | IFO medium 278 | AB032583 | (M62794) |
| '*Flexibacter flexilis* subsp. *pelliculosus*' | IFO 16028 | Lake shore, MN, USA | IFO medium 278 | AB032578 | |
| [*Flexibacter*] *filiformis* | IFO 15056$^T$ | Soil, Samoa | IFO medium 277 | AB032581 | (M58782) |
| [*Flexibacter*] *japonensis* | IFO 16041$^T$ | Soil, Japan | IFO medium 278 | AB032579 | |
| [*Flexibacter*] *litoralis* | ATCC 23117$^T$ | Sea-water aquarium outflow, CA, USA | ATCC medium 2 | AB034233 | (M58784) |
| [*Flexibacter*] *maritimus* | **ATCC 43398$^T$** | Red sea bream, Japan | 1/5 LBM | AB034229 | (D14023) |
| [*Flexibacter*] *maritimus* | **IFO 16015** | Black sea bream, Japan | 1/5 LBM | AB034228 | (D12667) |
| [*Flexibacter*] *ovolyticus* | **IAM 14318$^T$** | Halibut egg, Norway | 1/5 LBM | AB034230 | AB032506 |
| [*Flexibacter*] *ovolyticus* | **IFO 15992** | Halibut egg, Norway | 1/5 LBM | AB034231 | AB032507 |
| [*Flexibacter*] *ovolyticus* | **IFO 15993** | Halibut egg, Norway | 1/5 LBM | AB034232 | AB032508 |
| [*Flexibacter*] *sancti* | IFO 15057$^T$ | Soil, Argentina | IFO medium 272 | AB032582 | (M62795) |
| *Marinilabilia salmonicolor* | IFO 15948$^T$ | Marine mud | | (AB15032) | |
| '[*Microscilla*] *aggregans* subsp. *catalyticus*' | DSM 4133 | Sandy soil, AK, USA | 1/5 LBM | AB034237 | (M58791) |
| '[*Microscilla*] *furvescens*' | ATCC 23129 | Shore sand, Samoa | 1/5 LBM | AB034236 | |
| *Microscilla marina* | NCIMB 1400$^T$ | Marine aquarium outflow, CA, USA | 1/5 LBM | AB034238 | (M58793) |
| '*Microscilla sericea*' | DSM 4125 | Marine aquarium outflow, CA, USA | 1/5 LBM | AB032590 | |
| *Myroides odoratus* | IFO 14945$^T$ | Not known | IFO medium 275 | AB034239 | (M58777) |
| *Persicobacter diffluens* | IAM 14117$^T$ | Black sandy mud, India | 1/5 LBM | AB034219 | |
| *Polaribacter glomeratus* | **ATCC 43844$^T$** | Marine lake, Antarctica | 1/5 LBM | AB034235 | (M58775) |
| *Psychroflexus gondwanensis* | DSM 5423$^T$ | Organic lake, Antarctica | 1/5 LBM | AB034226 | (M92278) |
| *Sphingobacterium spiritivorum* | IAM 14210$^T$ | Uterus | | (AB15036) | |
| '[*Sporocytophaga*] *cauliformis*' | DSM 3656 | Lake water, Germany | DSM medium 67 | AB034240 | |

Rad) that had been suspended in, and washed with, USP buffer [8·0 M urea, 1·0 % (w/v) SDS and 0·24 M $NaH_2PO_4$ at pH 6·8]. Approximately 2 mg DNA in the USP buffer was loaded into this hydroxyapatite suspension and mixed gently. The suspension was centrifuged at 1870 ***g*** for 5 min at room temperature, and the buffer was discarded. The resulting pellet was washed once with USP buffer and twice with 0·2 M ammonium carbonate. The purified DNA sample was then eluted with 2 M ammonium carbonate (the eluate being dialysed against distilled water) and the DNA recovered by precipitation with 2-propanol. The DNA G+C content (mol %) was determined using HPLC (Suzuki *et al.*, 1999). DNA–DNA hybridization experiments were performed according to the method described by Ezaki *et al.* (1989). The hybridization reaction was performed at 38 °C in 2× SSC (1× SSC is 0·15 M NaCl plus 15 mM sodium citrate at pH 7·0) supplemented with 30 % (w/v) formamide.

**Sequencing of *gyrB* and 16S rDNA.** The *gyrB* gene sequence of each strain tested was determined as described previously (Suzuki *et al.*, 1999). The 16S rDNA gene sequences of our

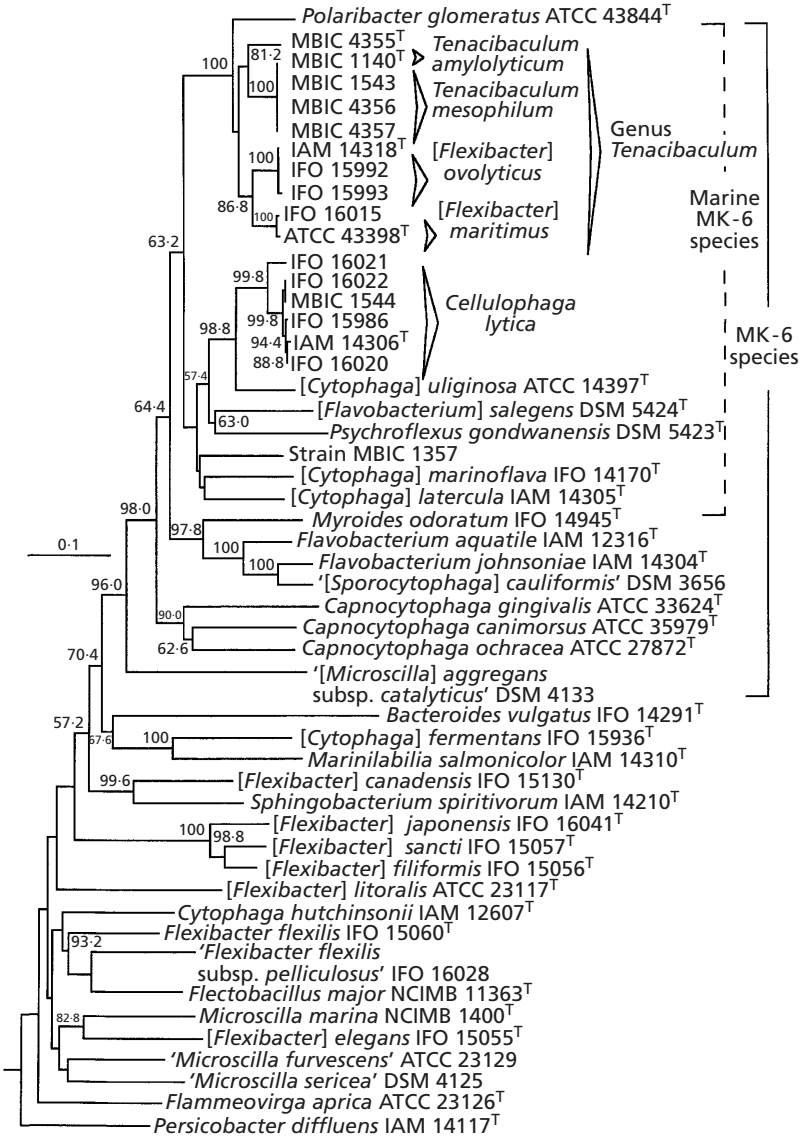


***Fig. 1.*** Phylogenetic tree of the *Cytophaga–Flavobacterium–Bacteroides* complex based on GyrB sequences, using the neighbour-joining method (Saitou & Nei, 1987). *Bacillus subtilis* was used as the outgroup. Number at each node indicates the percentage bootstrap value of 500 replicates. Bar, genetic distance of 0·1 calculated with the Dayhoff PAM model. '[*Microscilla*] *aggregans* subsp. *catalyticus*' DSM 4133 and all the strains located above it in the tree possess MK-6 as their major respiratory quinone.

isolates and of the strains of [*Flexibacter*] *ovolyticus* and *Cellulophaga lytica* were determined by PCR amplification and direct sequencing (Hiraishi, 1992). The BF primer (forward; 5′-AGAGTTTGATCCTGGCTCAG-3′, corresponding to positions 8–27 of *Escherichia coli* 16S rRNA) and the BR2 primer (reverse; 5′-TACGGTTACCTTGTTACGACTT-3′, positions 1513–1492), were designed according to the conserved sequences in the 5′- and 3′-terminal regions of the gene (Weisburg *et al*., 1991). The PCR was performed with Ampli*Taq* Gold DNA polymerase (Perkin Elmer) in a PCR buffer (Perkin Elmer) containing each dNTP at a concentration of 200 μM, $MgCl_2$ at 1·5 μM, each primer at 10 pM, and 2·5 U enzyme in a total volume of 50 μl. A total of 35 amplification cycles (denaturation at 95 °C for 45 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 1 min) were performed. The amplified DNA fragments were sequenced with a *Taq* DyeDeoxy Terminator Cycle sequencing kit (Perkin Elmer) and an ABI377 DNA sequencer (ABI). The primers used for sequencing were as follows: BF, BR2, 1m (5′-CTCCTACGGGAGGCAGCAGT-3′, positions 339–358 of *E. coli* 16S rRNA), 4m (5′-GTGTAGMRGTGAAATKCGTAGA-3′, positions 683–704), 6m (5′-ACAGGTGCTGCATGGCTGTCG-3′, positions 1044–1064), 3R (5′-TATTACCGCGGCTGCTGGCA-3′, positions 535–516) and 5R (5′-CCCCGTCAATTCATTTGAGT-3′, positions 928–909). These primers were designed for consensus regions of 16S rDNA (Weisburg *et al*., 1991).

**Phylogenetic analysis.** The nucleotide sequences of *gyrB* were translated to the amino acid sequences (GyrB), and these GyrB sequences were aligned using CLUSTAL W software (Thompson *et al*., 1994). Gaps were manually excluded from the aligned sequences. Evolutionary distances were computed with the PROTDIST program in the PHYLIP 3.572 package (Felsenstein, 1995) with the Dayhoff PAM matrix (Dayhoff *et al*., 1978). The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). A more detailed analysis of our isolates and their neighbouring species was based on the nucleotide sequences of *gyrB*. The *gyrB* sequences were aligned using CLUSTAL W software, the evolutionary distances were then computed with the DNADIST program in the PHYLIP 3.572 package with

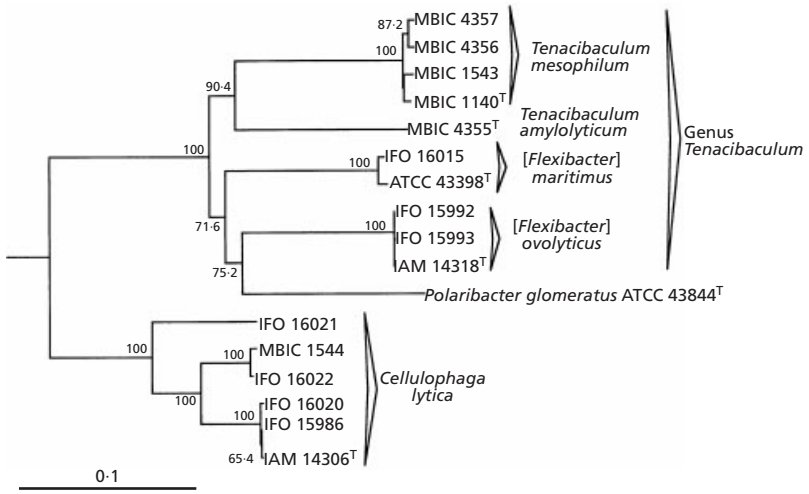


***Fig. 2.*** Phylogenetic tree of the genus *Tenacibaculum* and of *Cellulophaga lytica* strains based on *gyrB* nucleotide sequences, using the neighbour-joining method (Saitou & Nei, 1987). *Cytophaga uliginosa* ATCC 14397$^T$ was used as the outgroup. Number at each node indicates the percentage bootstrap value of 500 replicates. Bar, genetic distance of 0·1 ($K_{nuc}$).

the Kimura two-parameter model (Kimura, 1980), and the phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). The partial sequences (positions 111–1375 of the *E. coli* 16S rDNA sequence) of 16S rDNA were also aligned by using CLUSTAL W software. Regions which might contribute to incorrect alignment (positions 181–219, 457–478 and 838–850) were excluded.

To evaluate the phylogenetic trees, a bootstrap analysis with 500 sample replications was performed using the SEQBOOT and CONSENSE programs of the PHYLIP 3.572 package.

## RESULTS

### Phylogeny

A phylogenetic tree of the CFB complex, based on the amino acids sequences of GyrB, is shown in Fig. 1. The species containing menaquinone-6 (MK-6) formed one cluster.The species of the genus *Flexibacter* were located in various phylogenetic positions among the CFB complex. The GyrB sequence similarities between the *Flexibacter* species tested and *Flexibacter flexilis*, the type species of the genus were in the range 69·3 % ([*Flexibacter*] *maritimus*, [*Flexibacter*] *ovolyticus*, [*Flexibacter*] *sancti*) to 76·4 % ([*Flexibacter*] *elegans*) – values as low as those for species of other genera, such as *Cytophaga hutchinsonii* (77·6 %).

Among the MK-6-containing species, those from marine sources, except '[*Microscilla*] *aggregans* subsp. *catalyticus*' DSM 4133, formed a cluster. This group is therefore called the marine MK-6 group; all of our isolates are included in this group. Five of them, MBIC 1140$^T$, MBIC 1543, MBIC 4355$^T$ MBIC 4356 and MBIC 4357, were closely related to two generically misclassified species, [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus*, that exhibited high GyrB similarity (95·2 %) with each other. *Polaribacter glomeratus*, which had been isolated from an Antarctic marine lake, was the sister group to this cluster.

Strain MBIC 1544, isolated from a sponge collected in Japan, was positioned in a branch that comprised *Cellulophaga lytica*. The GyrB sequence of MBIC 1544 was identical to that of *Cellulophaga lytica* IFO 16022 and had 96·5–99·1 % similarity to the other strains of *Cellulophaga lytica*.

Strain MBIC 1357, isolated from a sponge collected in Palau, was positioned in the marine MK-6 group of the CFB complex, but the branching of this strain was deep and had no close neighbour.

The result of a more detailed phylogenetic analysis based on the nucleotide sequences of *gyrB* is shown in Fig. 2. This analysis used the *gyrB* sequences of [*Flexibacter*] *maritimus*, [*Flexibacter*] *ovolyticus*, *Cellulophaga lytica* and our isolates. The *gyrB* sequence similarity between the two strains of [*Flexibacter*] *maritimus* was 99·2 %, that between the three strains of [*Flexibacter*] *ovolyticus* was 99·9–100 %, while that between the six strains of *Cellulophaga lytica* was 88·5–99·9 %. Four isolates, MBIC 4357, MBIC 4356, MBIC 1543 and MBIC 1140$^T$, formed a cluster independent of the other related strains. Although their GyrB sequences were 100 % identical, some synonymous substitutions were observed in their *gyrB* sequences. The clustering of these strains was consistent with their geographical origin, two strains isolated in Palau (MBIC 4356 and MBIC 4357) forming one subcluster, but another subcluster which contains two strains isolated in Japan (MBIC 1140$^T$ and MBIC 1543) was not supported by bootstrap analysis.

The phylogenetic relationship among bacteria in the marine MK-6 group was also examined by analysis of their 16S rDNA sequences. The 16S rDNA-based tree is shown in Fig. 3; the branching patterns of each lineage in the 16S rDNA phylogram are in accordance with those in the GyrB phylogram. Strains MBIC 1140$^T$, MBIC 1543, MBIC 4356, MBIC 4357 and MBIC 4355$^T$ formed one consistent group neighbouring [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus*. The genus *Polaribacter*, which comprises several psychrophilic bacteria of Antarctic and Arctic origin, was a sister group to these bacteria.

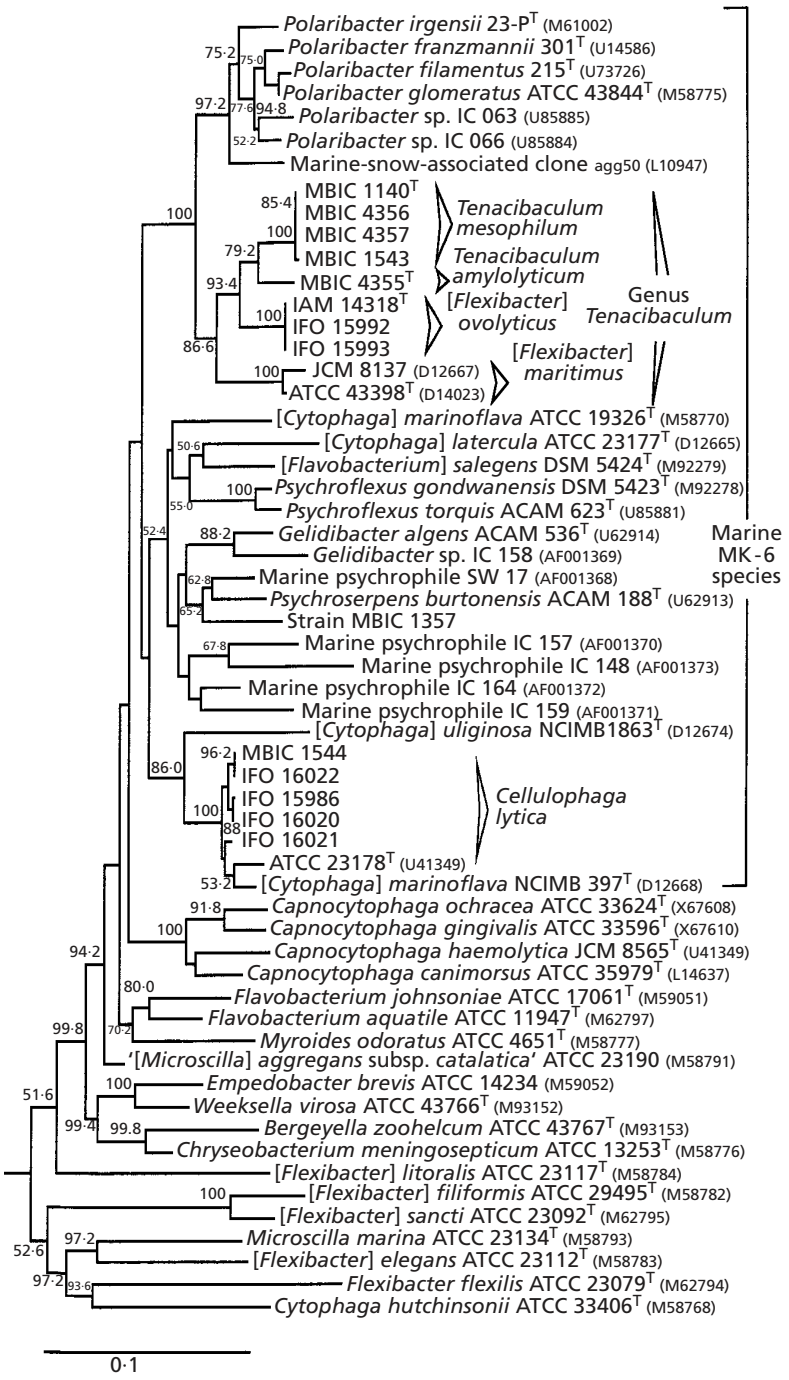


***Fig. 3.*** Phylogenetic tree of the *Cytophaga–Flavobacterium–Bacteroides* complex based on 16S rDNA sequences, using the neighbour-joining method (Saitou & Nei, 1987). *Chlorobium vibrioforme* (M62791) was used as the outgroup. Number at each node indicates the percentage bootstrap value of 500 replicates. Bar, genetic distance of 0·1 ($K_{nuc}$).

The other two isolates were also placed in the marine MK-6 group on the basis of the results of the 16S rDNA analysis. The MBIC 1357 strain isolated from a sponge collected from Palau showed limited relatedness to the psychrophilic genus *Psychroserpens*. However, strain MBIC 1357 was mesophilic and able to grow at 40 °C. The MBIC 1544 strain isolated from a sponge collected in Japan was located within the *Cellulophaga lytica* cluster. The 16S rDNA and *gyrB* sequences of MBIC 1544 were identical to those of IFO 16022.

## Phenotypic characterization

The five isolates constituting a single cluster in the dendrograms resulting from phylogenetic studies, MBIC 1140$^T$, MBIC 1543, MBIC 4355$^T$, MBIC 4356 and MBIC 4357, were studied to determine their phenotypic characteristics. They all produced bright-yellow colonies on 1/5 LBM agar and did not adhere to the agar plate. The irregularly spreading edges of their growth margins suggested the presence of gliding motility. After a 5 d incubation, the sizes of the colonies were between 35 and 60 mm in diameter,

***Table 2.*** Characteristics of species of the genus *Tenacibaculum*

Data are from this study and others (Wakabayashi *et al*., 1986; Hansen *et al*., 1992; Gosink *et al*., 1998). Abbreviations: +, positive test result; −, negative test result; V, test results vary between strains; ND, not determined; NG, no growth; W, weak result. All cells are rods, and the colony elevation for all four species was flat, and all four gave positive results for growth on tryptone and degradation of casein.

| Characteristic | *T. mesophilum* (*n* = 4) | *T. amylolyticum* (*n* = 1) | *T. ovolyticum* (*n* = 3) | *T. maritimum* (*n* = 2) |
|---|---|---|---|---|
| Cell size (μm) | 1·5–10 × 0·5 | 2–5 × 0·4 | 2–20 × 0·5 | 2–30 × 0·5 |
| Colony morphology: | | | | |
| Form | Circular or irregular with spreading edge | Circular with spreading edge | Regular edge | Uneven edge |
| Size at 5 d | 30–60 mm | 23–27 mm | ND | < 5 mm |
| Colour | Yellow | Yellow | Pale yellow | Pale yellow |
| Growth temp. | 15–40 | 20–35 | 4–25 | 15–34 |
| Optimal temp. | 28–35 | 27–30 | ND | 30 |
| Salinity range (%): | | | | |
| NaCl | 1–7 (1–10)* | 3 (W) | NG | NG |
| Sea water | 10–100 | 50–100 | 70–100 | 30–100 |
| pH range | 5·3–9·0 | 5·3–8·3 | 5·9–9·0 | 5·9–8·6 |
| Growth on: | | | | |
| Casamino acids | + | + | V | + |
| *N*-Ac-GlcN | − | − | − | −† |
| Sucrose | − | − | − | −† |
| D-Ribose | − | − | − | −† |
| DL-Aspartate | + | − | − | − |
| L-Proline | + | + | − | − |
| L-Glutamate | + | + | − | W (−†) |
| Degradation of: | | | | |
| Starch | − | + | − | − |
| Chitin | − | − | +‡ | − |
| Gelatin | + | + | + | − |
| Nitrate reduction | − | W | + | +§ |
| G+C content (mol%) | 31·6–32·0 | 30·9 | 30·3–32·0 | 31·3–32·5 |
| Type strain | MBIC 1140$^T$ | MBIC 4355$^T$ | IAM 14318$^T$ | ATCC 43398$^T$ |

* Strain MBIC 4356 is able to grow in a medium containing 10% (w/v) NaCl.

† There is a difference between the results of this study and those reported by Gosink *et al*. (1998).

‡ There is a difference between the results of this study and those reported by Hansen *et al*. (1992).

§ There is a difference between the results of this study and those reported by Wakabayashi *et al*. (1986).

except for those of MBIC 4355$^T$, which were between 23 and 27 mm in diameter. They all actively glided on glass and agar surfaces as observed using hanging drop preparations of liquid culture. Each of these isolates consisted of Gram-negative, flexirubin-negative, non-spore-forming, non-gas-vesicle-forming rods. The sphaeroplast-like round forms were rarely observed even after prolonged incubation of all isolates. The cell size of each strain is given in Table 2, and phase-contrast microphotographs of MBIC 1140$^T$ and MBIC 4355$^T$ are shown in Fig. 4.

Strain MBIC 4355$^T$ grew at temperatures between 20 and 35 °C, the optimum temperature being between 27 and 33 °C. The other four strains grew at temperatures between 15 and 40 °C, the optimum temperature being between 28 and 35 °C. These bacteria did not grow at ⩽ 6 °C or at ⩾ 45 °C. All but MBIC 4355$^T$ grew well in the LBM broth at pH 5·3–9·0, and strain MBIC 4355$^T$ grew well at pH 5·3–8·3. All of the isolates were obligate aerobes and no anaerobic growth was observed. All of the strains grew well on the 1/5 LBM medium containing 70% (v/v) sea water. Strain MBIC 4355$^T$ required sea water at a concentration higher than 50% (v/v) for sufficient growth, whereas the other strains required sea water at a concentration above 10% (v/v). The addition of 3% (w/v) NaCl to the 1/5 LBM medium weakly supported growth of strain MBIC 4355$^T$. The other strains grew well in 1/5 LBM medium supplemented with NaCl at 1–7% (w/v), whereas strain MBIC 4356 was able to grow in the 1/5 LBM medium containing 10% (w/v) NaCl.

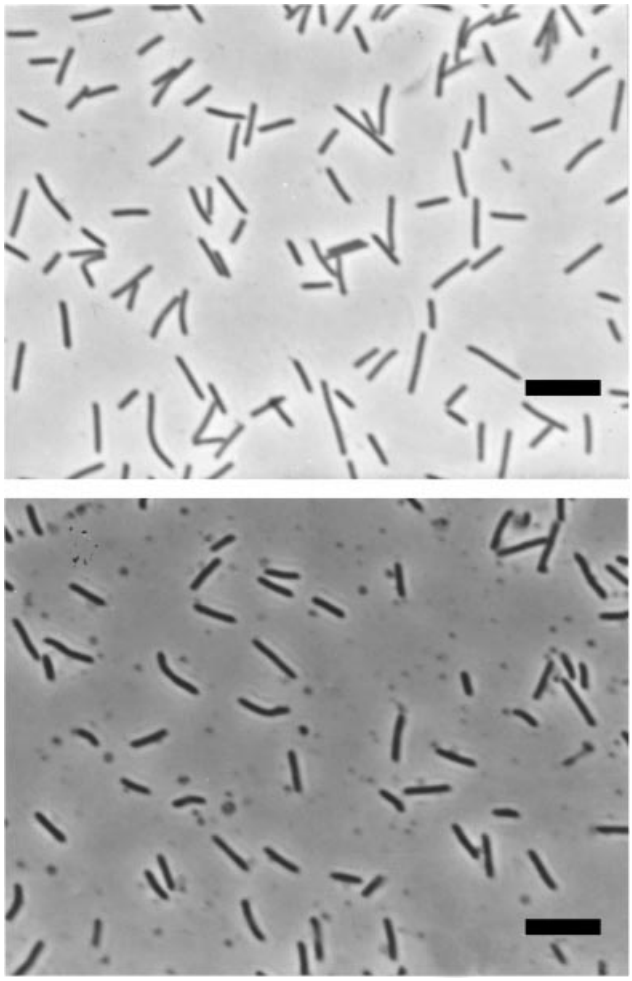

***Fig. 4.*** Phase-contrast microphotographs of (top) the sponge-associated strain MBIC 1140$^T$ and (bottom) the green-algae-associated strain MBIC 4355$^T$, grown on 1/5 LBM agar at 20 °C for 3 d. Bars, 5 μm.

The five isolates and [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus* strain shared many characteristics. They all grew with tryptone and Casamino acids, but did not grow on ethanol, methanol, glycerol, L-tartrate, DL-malate, α-ketoglutarate, fumarate, citrate, succinate, pyruvate, propionate, acetate, benzoate or L-leucine. They all degraded casein, tyrosine, Tween 80 and DNA, but not cellulose, aesculin and agar. They all produced oxidase and catalase. They were all unable to grow anaerobically. The characteristics that may be used to differentiate the isolates from each other and from [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus* are listed in Table 2. Hansen *et al.* (1992) have reported that [*Flexibacter*] *ovolyticus* did not degrade chitin. In this study, however, the [*Flexibacter*] *ovolyticus* type strain, as well as two other strains, degraded chitin after prolonged incubation (10 d). Wakabayashi *et al.* (1986) reported that [*Flexibacter*] *maritimus* degraded gelatin, but, in this study, the type strain (ATCC 43398$^T$) did not degrade gelatin. Some properties of [*Flexibacter*] *maritimus* were different from those reported by Gosink *et al.* (1998) (see Table 2). These discrepancies could have been due to a difference in the growth conditions: in this study, the ability to grow on various carbon substrates was tested by using a basal medium supplemented with yeast extract at a concentration of 0·05 g l$^{-1}$ (the previous study involved a medium supplemented with amino acids). The growth on amino acids was not affected when inorganic nitrogen sources were omitted from the basal medium and nitrate or ammonium could not be used as nitrogen sources (data not shown). This observation indicates that these bacteria could utilize certain amino acids as sole carbon and nitrogen sources, and require an organic nitrogen source for growth.

### Chemotaxonomic characteristics

All of the isolates, including MBIC 1357 and MBIC 1544, possessed MK-6 as the respiratory quinone system. All isolates and the strains of [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus* contained zeaxanthin as the major carotenoid pigment, contained β-cryptoxanthin and β-carotene as minor components, but did not contain flexirubin-type pigments.

### Genotypic characteristics

The G+C compositions of the isolates were in the range 31–32 mol%. The DNA–DNA hybridization results among our isolates and their related species are shown in Table 3. The MBIC 1140$^T$, MBIC 1543, MBIC 4356 and MBIC 4357 strains showed 86–95% DNA relatedness to each other and 21–35% DNA relatedness to [*Flexibacter*] *ovolyticus* and [*Flexibacter*] *maritimus*. Strain MBIC 4355$^T$ did not show high levels of DNA relatedness to the other strains used.

The levels of DNA–DNA relatedness among the strains of *Cellulophaga lytica* and between this species and strain MBIC 1544 are indicated in Table 4. Strain MBIC 1544 showed homology of 68–92% with respect to the strains of *Cellulophaga lytica*. This shows that MBIC 1544 is a member of the *Cellulophaga lytica* species. The five other strains of *Cellulophaga lytica* tested showed homology of more than or nearly 70% to each other. The levels of relatedness between IFO 15986, IFO 16020 and IAM 14306$^T$ were very high (> 99%). The homology between strains MBIC 1544 and IFO 16022 was also high (95%). IFO 16021 showed moderate homology (70–76%) with respect to the other strains of *Cellulophaga lytica*. This shows that while IFO 16021 is a *Cellulophaga lytica* strain, it is less closely related to the other strains in this species.

## DISCUSSION

The phylogenetic structures of the CFB complex based on the GyrB sequences and on the 16S rDNA sequences were almost equivalent. The marine species possessing MK-6 were clustered in one lineage, with the exception of '[*Microscilla*] *aggregans* subsp. *cata-*

***Table 3.*** Percentage DNA–DNA hybridization between strains of the genus *Tenacibaculum* and *Polaribacter glomeratus*

Each DNA–DNA hybridization value is the mean of the values from three replicates; error values shown are standard deviations.

| Strain | G+C content (mol%) | DNA–DNA reassociation (%) with: | | | | | |
|---|---|---|---|---|---|---|---|
| | | MBIC 1140$^T$ | MBIC 4356 | MBIC 4355$^T$ | IAM 14318$^T$ | ATCC 43398$^T$ | ATCC 43844$^T$ |
| *Tenacibaculum mesophilum* MBIC 1140$^T$ | 31·8 | 100 | 95±5 | 25±4 | 35±6 | 25±8 | 18±4 |
| *Tenacibaculum mesophilum* MBIC 1543 | 31·6 | 91±3 | 92±4 | 23±3 | 32±3 | 24±7 | 18±3 |
| *Tenacibaculum mesophilum* MBIC 4356 | 32·0 | 88±1 | 100 | 22±4 | 31±4 | 23±7 | 17±3 |
| *Tenacibaculum mesophilum* MBIC 4357 | 32·0 | 86±2 | 86±2 | 22±4 | 31±4 | 23±7 | 17±3 |
| *Tenacibaculum amylolyticum* MBIC 4355$^T$ | 30·9 | 16±5 | 16±2 | 100 | 14±2 | 12±3 | 10±1 |
| [*Flexibacter*] *ovolyticus* IAM 14318$^T$ | 30·3 | 27±8 | 21±7 | 17±2 | 100 | 19±5 | 14±2 |
| [*Flexibacter*] *maritimus* ATCC 43398$^T$ | 31·6 | 25±7 | 21±8 | 18±3 | 23±3 | 100 | 16±4 |
| *Polaribacter glomeratus* ATCC 43844$^T$ | 33·2 | 17±3 | 16±2 | 11±2 | 14±2 | 12±4 | 100 |
| *Cellulophaga lytica* IAM 14306$^T$ | 32·7 | 10±2 | 10±1 | 8±1 | 9±1 | 8±2 | 9±1 |

***Table 4.*** Percentage DNA–DNA hybridization between strains of *Cellulophaga lytica*

Each DNA–DNA hybridization value is the mean of the values from three replicates; error values shown are standard deviations.

| Strain | G+C content (mol%) | DNA–DNA reassociation (%) with: | | | |
|---|---|---|---|---|---|
| | | IAM 14306$^T$ | IFO 16022 | MBIC 1544 | IFO 16021 |
| *Cellulophaga lytica* IAM 14306$^T$ | 32·7 | 100 | 84±5 | 74±2 | 75±4 |
| *Cellulophaga lytica* IFO 15986 | 34·0 | 103±11 | 83±4 | 77±2 | 74±2 |
| *Cellulophaga lytica* IFO 16020 | 34·6 | 100±5 | 83±1 | 76±3 | 76±1 |
| *Cellulophaga lytica* IFO 16022 | ND | 80±5 | 100 | 92±3 | 77±2 |
| *Cellulophaga lytica* MBIC 1544 | 32·8 | 84±5 | 105±7 | 100 | 80±5 |
| *Cellulophaga lytica* IFO 16021 | 33·0 | 72±2 | 71±1 | 68±2 | 100 |
| *Tenacibaculum mesophilum* MBIC 1140$^T$ | 31·8 | 17±1 | 16±1 | 14±1 | 18±1 |

*lyticus*'. However, such clustering of marine strains has not been apparent in previous studies: the marine strains containing MK-6 were previously divided into several clades (Maidak *et al.*, 1999; Gosink *et al.*, 1998). In those studies, the maximum-likelihood method was used for the phylogenetic analyses. Thus, the different method of analysis might be the reason for the difference between our results and theirs. In both the GyrB- and the 16S rDNA-based trees constructed in this study, the bootstrap values of the marine MK-6 group were not particularly high (less than 50% in both analyses). Therefore, the single lineage of the marine strains containing MK-6 should not be overemphasized. Further studies are needed to clarify the phylogenetic structure of the marine MK-6 group.

Two different 16S rDNA sequences from the type strain of *Cytophaga marinoflava* are deposited in the GenBank database. One (M58770) forms a deep branch from the lineage of [*Cytophaga*] *latercula*, [*Flavobacterium*] *salegens* and the *Psychroflexus* genus. The other one (D12668) is located in the cluster of *Cellulophaga lytica*. The results of the present GyrB analysis indicate that [*Cytophaga*] *marinoflava* IFO 14170$^T$ was not clustered in the *Cellulophaga lytica* group but was related to [*Cytophaga*] *latercula* IAM 14305$^T$. The reason why two different 16S rDNA sequences of the type strain of *Cytophaga marinoflava* are registered is unknown. Our data support the M58870 sequence as that of [*Cytophaga*] *marinoflava*.

The *gyrB* sequence similarity among the strains used in the DNA–DNA hybridization experiment is indicated in Tables 3 and 4 and summarized in Fig. 5. Good correlation is apparent between the DNA–DNA hybridization values and the *gyrB* sequence similarity. The proposed limit for a species identity, the DNA reassociation value of 70% (Wayne *et al.*, 1987), corresponds to a *gyrB* sequence similarity of approximately 88·8%. On the other hand, the *gyrB* sequence similarity between strains of different species is less than 83·5%. The range of *gyrB* sequence similarity within the same species observed in the present study is almost identical to that observed in genomic species of the *Acinetobacter* genus (Yamamoto *et al.*, 1999). The correlation between the DNA–DNA hybridization value and the 16S rDNA sequence similarity was less

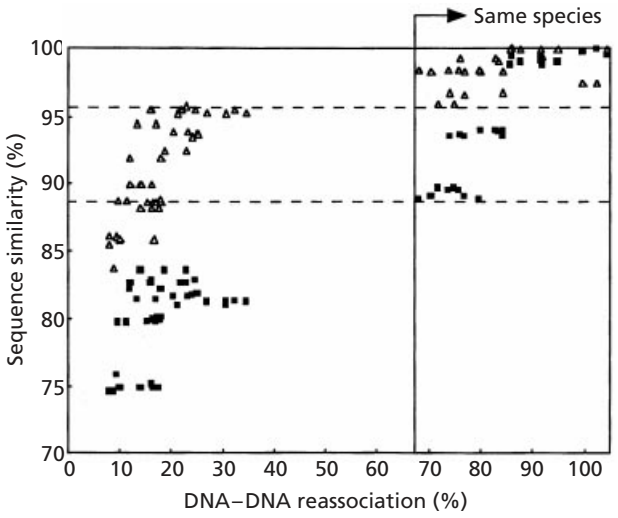


***Fig. 5.*** Comparison of sequence similarities and DNA–DNA reassociation values. All available combinations between strains of *Tenacibaculum* species, *Polaribacter glomeratus* and *Cellulophaga lytica* are plotted. Open triangles, 16S rDNA sequence similarity; filled squares, *gyrB* sequence similarity.

obvious. In this study, the lowest 16S rDNA sequence similarity between strains of the same species was 95·9 % (IAM 14306$^T$ and IFO16021), and the highest 16S rDNA similarity between strains of different species was 95·5 % (MBIC 1140$^T$ and MBIC 4355$^T$). Likewise, 16S rDNA similarity between *Cellulophaga lytica* (IAM 14306$^T$) and *Cellulophaga fucicola* NN 015860$^T$ (AJ005973, which was not used in this study) was 95·9 %. This value is the same to that of an intra-species comparison (IAM 14306$^T$ and IFO 16021). Thus, it was difficult to set a threshold value for 16S rDNA similarity for species identity.

In the actinomycetes, Kasai *et al.* (2000) have shown that a genetic distance of approximately 0·014 for *gyrB* would correspond to the 70 % DNA homology value within the *Micromonospora* genus. The genetic variation of *gyrB* within a species of the *Micromonospora* genus is very small in comparison with our *gyrB* data for strains in the CFB complex and the *Acinetobacter* strains. The reason for such a difference is not clear at present, but it is well known that the chromosome of the actinomycetes is unstable and often undergoes substantial deletion or rearrangement (Volff & Altenbuchner, 1998). Such deletion or rearrangement may reduce the degree of DNA–DNA hybridization much more significantly than the substitution of a single nucleotide.

Since the phylogenetic analysis based on the nucleotide sequences of *gyrB* seems to be more suitable than 16S rDNA for taxonomic purposes and for the identification of bacteria at the species level, it is desirable to establish the relationship between the DNA reassociation value and *gyrB* similarity. We are planning to carry out similar analyses with more bacterial strains.

On the other hand, genus identification by DNA–DNA hybridization seems to be difficult: there are some examples for which intergeneric *gyrB* sequence similarities are as high as intrageneric values (IAM 14318$^T$ and ATCC 43844$^T$, ATCC 43398$^T$ and ATCC 43844$^T$). Also, DNA–DNA hybridization values between strain MBIC 4355$^T$ and other strains of the genus *Tenacibaculum* are as low as intergeneric values. In contrast, the intrageneric 16S rDNA sequence similarities are always higher than intergeneric ones in this study. These results indicated that 16S rDNA sequence similarities are suitable for consideration at the generic level.

Previous 16S rRNA studies of the CFB complex (Woese *et al.*, 1990; Bernerdet *et al.*, 1996; Nakagawa & Yamasato, 1996; Sly *et al.*, 1999) had demonstrated that *Flexibacter flexilis*, the type species of the genus *Flexibacter*, is isolated on an independent lineage. Our analysis based on the GyrB sequences confirms that the genus *Flexibacter* is polyphyletic and should be thoroughly emended. The genus should be restricted to its type species, and all other species previously assigned to the genus should be reclassified in other or new genera. The marine bacterial strains that we isolated from sponge samples and from the surfaces of macro-algae collected in Palau and Japan were found to be located in three distinct taxonomic positions within the marine MK-6 group in the CFB complex. Strain MBIC 1544 was identified as *Cellulophaga lytica*, because of its high DNA homology with other strains belonging to this species. Strain MBIC 1357 formed an independent lineage within the marine MK-6 group, according to the phylogenetic analysis based on *gyrB* and 16S rDNA. Some other close relatives are needed to define the taxonomic status of this bacterium. The other five strains formed a tight cluster with [*Flexibacter*] *ovolyticus* and [*Flexibacter*] *maritimus* and shared many common characteristics of their morphology, physiology and chemotaxomony with these species. The bacteria of this group are phylogenetically related to the members of the genus *Polaribacter*. Although their chemotaxonomic characteristics are similar to those of the genus *Polaribacter*, no species of the genus *Polaribacter* glide, and their cell-mass colour is orange to pink. On the other hand, our five isolates, as well as [*Flexibacter*] *ovolyticus* and [*Flexibacter*] *maritimus* are yellow in colour and exhibit gliding motility. Furthermore, in contrast to these rod-shaped species, *Polaribacter* strains were irregular in shape. Whilst the species isolated do not form gas vesicles, three out of four species of the genus *Polaribacter* do form such vesicles. The present results allow us to conclude that MBIC 1140$^T$, MBIC 1543, MBIC 4356, MBIC 4357, MBIC 4355$^T$, [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus* should be assigned to a novel genus, for which we propose the name *Tenacibaculum* gen. nov. Some characteristics useful for discriminating between this genus and its close relatives are shown in Table 5. The DNA–DNA hybridization data showed that strains MBIC 1140$^T$,

***Table 5.*** Differential characteristics for the genus *Tenacibaculum* and other marine species in the family *Flavobacteriaceae*

Species: 1, *Tenacibaculum mesophylum*; 2, *Tenacibaculum amylolyticam*; 3, *Tenacibaculum ovolyticum*; 4, *Tenacibaculum maritimum*; 5, *Polaribacter glomeratus*; 6, *Polaribacter franzmannii*; 7, *Polaribacter filamentus*; 8, *Polaribacter irgensii*; 9, *Psychroserpens burtonensis*; 10, *Gelidibacter algens*; 11, *Psychroflexus torquis*; 12, *Psychroflexus gondwanensis*; 13, *Cellulophaga lytica*; 14, *Cellulophaga baltica*; 15, *Cellulophaga fucicola*; 16, *Cytophaga latercula*; 17, *Cytophaga marinoflava*; 18, *Cytophaga uliginosa*; 19, *Flavobacterium salegens*. Data are from this study and others (Wakabayashi *et al.*, 1986; Hansen *et al.*, 1992; Gosink *et al.*, 1998; Dobson *et al.*, 1993; Colwell *et al.*, 1966; Johansen *et al.*, 1999; Bowman *et al.*, 1997a, 1998; Lewin, 1969). Abbreviations: V, variable; W, weak reaction; ND, not determined; NG, no growth.

| Character | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell morphology | Rod | Rod | Rod | Rod | Spiral | Irregular rod | Filamentous | Filamentous | Ring, helical | Rod | Filamentous | Rod | Rod | Rod | Rod | Rod | Rod | Rod | Rod |
| Colony colour | Yellow | Yellow | Yellow | Yellow | Orange | Orange | Salmon | Orange | Yellow | Yellow | Orange | Orange | Yellow | Yellow | Yellow | Orange | Yellow | Yellow | Yellow |
| Gliding motility | + | + | + | + | − | − | − | − | − | + | + | − | + | + | + | − (−/+*) | + | + | − |
| Growth temp. (°C) | 15–40 | 20–35 | 4–25 | 15–34 | 4–21 | 4–10 | 4–15 | −1·5–10 | < 20 | < 25 | < 20 | < 30 | < 40 | 2–30 | 2–30 | < 35 | 4–30 | 15–40 | < 25 |
| Growth with 1% peptone | + | + | + | + | − | ND | ND | ND | − | + | − | ND | + | ND | ND | + | + | + | ND |
| Organic N requirement | + | + | + | + | − | ND | ND | ND | + | + | + | − | − | ND | ND | − | − | − | − |
| Degradation of: | | | | | | | | | | | | | | | | | | | |
| Casein | + | + | + | + | − | − | − | − | + | V | ND | − | − | + | − | +† | − | + | − |
| Starch | − | + | − | − | + | W | W | W | − | + | + | + | + | + | + | +† | + | + | + |
| Gelatin | + | + | + | − | − | + | W | − | V | V | + | − | + | + | − | −† | ND | −† | + |
| Agar | − | − | − | − | ND | ND | ND | ND | − | − | − | − | + | + | + | + | − | + | − |
| Oxidase | + | + | + | + | + | + | − | W | − | − | + | + | + | − | − | + | + | + | + |
| Nitrate reduction | − | W | + | + | + | − | − | − | − | − | − | − | − | ND | ND | +(−/+*) | + | + | + |
| G+C content (mol%) | 31·6–32·0 | 30·9 | 31–32 | 31–32 | 32–33·2 | 32 | 32 | 31 | 27–29 | 36–38 | 32–33 | 35–39 | 33 | 33 | 32·4 | 34 | 37 | 42 | 39–41 |

* Previously reported results differ.

† There is a difference between the results of this study and previously reported results.

MBIC 1543, MBIC 4356 and MBIC 4357 formed a tight genomic species whose members were more than 86% related to each other and less than 27% related to all other strains studied. Strain MBIC 4355$^T$ showed only low levels of DNA homology with all other strains studied. We therefore conclude that these strains constitute two new bacterial species in the genus *Tenacibaculum*, distinct from [*Flexibacter*] *maritimus* and [*Flexibacter*] *ovolyticus*. The new genus and the four species it contains are described below. Differential characteristics for the species are listed in Table 2.

### Description of *Tenacibaculum* gen. nov.

*Tenacibaculum* (Te.na.ci.ba′cu.lum. L. adj. n. *tenax* holding fast; L. neut. n. *baculum* stick; N.L. neut. n. *Tenacibaculum* rod-shaped bacterium which adheres to the surfaces of marine organisms).

Gram-negative. Cells are rod-shaped, 1·5–30 μm long and 0·4–0·5 μm wide. Ring-shaped cells and gas vesicles are not formed. Produce a yellow pigment which is mainly zeaxanthin. Flexirubin-type pigment is absent. Spores are not formed. Cells are non-flagellated and are motile by gliding. Strictly aerobic heterotrophs. Produce catalase and oxidase. Major respiratory quinone is MK-6. G+C content of the DNA is 31–33 mol%. Member of the family *Flavobacteriaceae*. All strains were isolated from a marine environment and grow well on media containing sea water. One species can grow actively on media containing 1–7% NaCl (w/v). Type species of the genus is *Tenacibaculum maritimum*.

### Description of *Tenacibaculum maritimum* (Wakabayashi *et al.* 1986) gen. nov., comb. nov.

The description is identical to that given for *Flexibacter maritimus* by Wakabayashi *et al.* (1986). The type strain is NCIMB 2514$^T$ and was isolated from a diseased red sea bream fingerling in Japan.

### Description of *Tenacibaculum ovolyticum* (Hansen *et al.* 1992) gen. nov., comb. nov.

The description is identical to that given for *Flexibacter ovolyticus* by Hansen *et al.* (1992). Type strain is NCIMB 13127$^T$ and was isolated from the adherent epiflora of halibut eggs in Western Norway.

### Description of *Tenacibaculum mesophilum* gen. nov., sp. nov.

*Tenacibaculum mesophilum* [me.so.phi′lum. Gr. n. *mesos* middle; Gr. adj. *philos* loving; N.L. n. adj. *mesophilum* middle (temperature)-loving, i.e. mesophilic].

Gram-negative. Cells are rod-shaped, 1·5–10 μm long and 0·5 μm wide. Colonies on 1/5 LBM medium are circular with a spreading and undulating margin, a flat elevation, a 40–50 mm diameter after 5 d incubation and are pigmented bright yellow. Requires 1/10 strength of sea water or 1% (w/v) NaCl for growth. Mesophilic: growth occurs at 15–40 °C, optimal growth occurring at 28–35 °C in liquid media. No growth occurs at temperatures at or below 10 °C and at or above 45 °C. Growth occurs at pH 5·3–9·0 in liquid media. Nitrate is not reduced. All strains hydrolyse casein, tyrosine, gelatin, Tween 80 and DNA. Growth occurs on peptone, tryptone, Casamino acids, DL-aspartate, L-proline and L-glutamate as the sole carbon and nitrogen sources. G+C content of the DNA is 31–32 mol% (determined by HPLC). Type strain is MBIC 1140$^T$ (= IFO 16307$^T$ = DSM13764$^T$), isolated from the sponge *Halichondria okadai*, which was collected in Numazu, Japan.

### Description of *Tenacibaculum amylolyticum* gen. nov., sp. nov.

*Tenacibaculum amylolyticum* (am.y.lo.ly′ti.cum. Gr. n. *amylum* starch; Gr. adj. *lyticos* loosening, dissolving; N.L. adj. *lyticus* dissolving; N.L. neut. adj. *amylolyticum* starch-dissolving).

Gram-negative. Cells are rod-shaped, 2–5 μm in length and 0·4 μm in width. Colonies on 1/5 LBM medium are circular with a spreading and undulating margin, a flat elevation, a 20–30 mm diameter after 5 d incubation and are pigmented bright yellow. Requires at least 1/2 strength sea water for sufficient growth. Mesophilic: growth occurs at 19–35 °C, optimal growth occurring at approximately 30 °C in liquid media. No growth occurs at temperatures at or below 15 °C and at or above 40 °C. Growth occurs at pH 5·3–8·3 in liquid media. Nitrate is weakly reduced. Hydrolyses casein, starch, tyrosine, gelatin, Tween 80 and DNA. Growth occurs on peptone, tryptone, Casamino acids, L-proline and L-glutamate as the sole carbon and nitrogen sources. G+C content of the DNA is 31 mol% (determined by HPLC). The type and only strain is MBIC 4355$^T$ (= IFO 16310$^T$ = DSM 13766$^T$), isolated from the green alga *Avrainvillea riukiuensisi*, which was collected in Palau.

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## REFERENCES

**Bernardet, J.-F. (1998).** *Cytophaga*, *Flavobacterium*, *Flexibacter* and *Chryseobacterium* infections in cultured marine fish. *Fish Pathol* **33**, 229–238.

**Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996).** Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and

proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.

**Bolinches, J., Lemos, M. L. & Barja, J. L. (1988).** Population dynamics of heterotrophic bacterial communities associated with *Fucus vesiculosus* and *Ulva rigida* in an estuary. *Microb Ecol* **15**, 345–357.

**Bowman, J. P., McCammon, S. A., Brown, J. L., Nichols, P. D. & McMeekin, T. A. (1997a).** *Psychroserpens burtonensis* gen. nov., sp. nov., and *Gelidibacter algens* gen. nov., sp. nov., psychrophilic bacteria isolated from Antarctic lacustrine and sea ice habitats. *Int J Syst Bacteriol* **47**, 670–677.

**Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S. & McMeekin, T. A. (1997b).** Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microbiol* **63**, 3068–3078.

**Bowman, J. P., McCammon, S. A., Lewis, T., Skerratt, J. H., Brown, J. L., Nichols, D. S. & McMeekin, T. A. (1998).** *Psychroflexus torquis* gen. nov., sp. nov., a psychrophilic species from Antarctic sea ice, and reclassification of *Flavobacterium gondwanense* (Dobson et al. 1993) as *Psychroflexus gondwanense* gen. nov., comb. nov. *Microbiology* **144**, 1601–1609.

**Clayton, R. A., Sutton, G., Hinkle, P. S., Jr, Bult, C. & Fields, C. (1995).** Intraspecific variation in small-subunit rRNA sequences in GenBank: why single sequences may not adequately represent prokaryotic taxa. *Int J Syst Bacteriol* **45**, 595–599.

**Colwell, R. R., Citarella, R. V. & Chen, P. K. (1966).** DNA base composition of *Cytophaga marinoflava* n. sp. determined by buoyant density measurement in cesium chloride. *Can J Microbiol* **12**, 1099–1103.

**Dayhoff, M. O., Schwartz, R. M. & Orcutt, B. C. (1978).** A model of evolutionary change in proteins, In *Atlas of Protein Sequence and Structure*, vol. 5, suppl. 3. pp. 345–352. Edited by M. O. Dayhoff. Washington, DC: National Biomedical Research Foundation.

**Dobson, S. J., Colwell, R. R., McMeekin, T. A. & Franzmann, P. D. (1993).** Direct sequencing of the polymerase chain reaction-amplified 16S rRNA gene of *Flavobacterium gondwanense* sp. nov. and *Flavobacterium salegens* sp. nov., two new species from a hypersaline Antarctic lake. *Int J Syst Bacteriol* **43**, 77–83.

**Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989).** Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

**Fautz, E. & Reichenbach, H. (1980).** A simple test for flexirubin-type pigments. *FEMS Microbiol Lett* **8**, 87–91.

**Felsenstein, F. (1995).** PHYLIP (Phylogeny Inference Package) version 3.57c. Seattle: University of Washington, Seattle, WA, USA.

**Fox, G. E., Wisotzkey, J. D. & Jurtshuk, P. J., Jr (1992).** How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int J Syst Bacteriol* **42**, 166–170.

**Gosink, J. J., Woese, C. R. & Staley, J. T. (1998).** *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga–Flavobacterium–Bacteroides* group and reclassification of '*Flectobacillus glomeratus*' as *Polaribacter glomeratus* comb. nov. *Int J Syst Bacteriol* **48**, 223–235.

**Hansen, G. H., Bergh, Ø., Michaelsen, J. & Knappskog, D. (1992).** *Flexibacter ovolyticus* sp. nov., a pathogen of eggs and larvae of Atlantic halibut, *Hippoglossus hippoglossus* L. *Int J Syst Bacteriol* **42**, 451–458.

**Hanzawa, N., Singleton, F. L., Hamada, E., Colwell, R. R. & Miyachi, S. (1993).** Isolation and characterization of marine bacterium producing a seed-germination inhibitor. *J Mar Biotechnol* **1**, 105–108.

**Hiraishi, A. (1992).** Direct automated sequencing of 16S rDNA amplified by polymerase chain reaction from bacterial cultures without DNA purification. *Lett Appl Microbiol* **15**, 210–213.

**Johansen, J. E., Nielsen, P. & Sjøholm, C. (1999).** Description of *Cellulophaga baltica* gen. nov., sp. nov. and *Cellulophaga fucicola* gen. nov., sp. nov. and reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 1231–1240.

**Kasai, H., Tamura, T. & Harayama, S. (2000).** Intrageneric relationships among *Micromonospora* species deduced from *gyrB*-based phylogeny and DNA relatedness. *Int J Syst Evol Microbiol* **50**, 127–134.

**Kimura, M. (1980).** A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

**Lewin, R. A. (1969).** A classification of flexibacter. *J Gen Microbiol* **58**, 189–206.

**Lewin, R. A. & Lounsbery, D. M. (1969).** Isolation, cultivation and characterization of flexibacteria. *J Gen Microbiol* **58**, 145–170.

**Maeda, T., Murakami, M., Ohsugi, S., Furushita, M., Mitsutani, A. & Shiba, T. (1998).** Perspectives of the development of 16S rDNA probe specific for algicidal and/or algal-lytic gliding bacteria. *Fish Sci* **64**, 861–865.

**Maidak, B. L., Cole, J. R. & Parker, C. T., Jr & 11 other authors (1999).** A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Res* **27**, 171–173.

**Nakagawa, Y. & Yamasato, K. (1993).** Phylogenetic diversity of the genus *Cytophaga* revealed by 16S rRNA sequencing and menaquinone analysis. *J Gen Microbiol* **139**, 1155–1161.

**Nakagawa, Y. & Yamasato, K. (1996).** Emendation of the genus *Cytophaga* and transfer of *Cytophaga agarovorans* and *Cytophaga salmonicolor* to *Marinilabilia* gen. nov.: phylogenetic analysis of the *Flavobacterium-Cytophaga* complex. *Int J Syst Bacteriol* **46**, 599–603.

**Nakanishi, K., Nishijima, M., Nishimura, M., Kuwano, K. & Saga, N. (1996).** Bacteria that induce morphogenesis in *Ulva pertusa* (Chlorophyta) grown under axenic conditions. *J Phycol* **32**, 479–482.

**Ochman, H. & Wilson, A. C. (1987).** Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. *J Mol Evol* **26**, 74–86.

**Olsen, G. J. & Woese, C. R. (1993).** Ribosomal RNA: a key to phylogeny. *FASEB J* **7**, 113–123.

**Perry, L. B. (1973).** Gliding motility in some non-spreading flexibacteria. *J Appl Bacteriol* **36**, 227–232.

**Pinhassi, J., Zweifel, U. L. & Hagström, Å. (1997).** Dominant marine bacterioplankton species found among colony-forming bacteria. *Appl Environ Microbiol* **63**, 3359–3366.

**Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

**Shah, H. N. & Collins, M. D. (1988).** Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and

*Bacteroides endodontalis* in a new genus, *Porphyromonas*. *Int J Syst Bacteriol* **38**, 128–131.

**Shiba, T. & Taga, N. (1980).** Heterotrophic bacteria attached to seaweeds. *J Exp Mar Biol Ecol* **47**, 251–258.

**Sly, L. I., Taghavi, M. & Fegan, M. (1999).** Phylogenetic position of *Chitinophaga pinensis* in the *Flexibacter–Bacteroides–Cytophaga* phylum. *Int J Syst Bacteriol* **49**, 479–481.

**Smibert, R. M. & Krieg, N. R. (1981).** General characterization. In *Manual of Methods for General Bacteriology*, pp. 409–443. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.

**Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

**Suzuki, M., Nakagawa, Y., Harayama, S. & Yamamoto, S. (1999).** Phylogenetic analysis of genus *Marinilabilia* and related bacteria based on the amino acid sequences of GyrB and emended description of *Marinilabilia salmonicolor* with *Marinilabilia agarovorans* as its subjective synonym. *Int J Syst Bacteriol* **49**, 1551–1557.

**Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

**Volff, J.-N. & Altenbuchner, J. (1998).** Genetic instability of the Streptomyces chromosome. *Mol Microbiol* **27**, 239–246.

**Wakabayashi, H., Hikida, M. & Masumura, K. (1986).** *Flexibacter maritimus* sp. nov., a pathogen of marine fishes. *Int J Syst Bacteriol* **36**, 396–398.

**Wayne, L. G., Brenner, D. J. & Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

**Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991).** 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

**Woese, C. R., Yang, D., Mandelco, I. & Stetter, K. O. (1990).** The *Flexibacter-Flavobacter* connection. *Syst Appl Microbiol* **13**, 161–165.

**Yamamoto, S. & Harayama, S. (1995).** PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104–1109.

**Yamamoto, S. & Harayama, S. (1996).** Phylogenetic analysis of *Acinetobacter* strains based on the nucleotide sequences of *gyrB* genes and on the amino acids sequences of their products. *Int J Syst Bacteriol* **46**, 506–511.

**Yamamoto, S., Bouvet, P. J. M. & Harayama, S. (1999).** Phylogenetic structures of the genus *Acinetobacter* based on *gyrB* sequences: comparison with the grouping by DNA–DNA hybridization. *Int J Syst Bacteriol* **49**, 87–95.