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

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The detailed phylogenetic relationships for genus *Marinilabilia* and related taxa were analysed by using DNA gyrase B subunit gene (*gyrB*) sequences. Anaerobic bacteria in the *Cytophaga*–*Flavobacterium*–*Bacteroides* phylum, namely genera *Marinilabilia*, *Bacteroides*, *Rikenella*, *Prevotella* and *Porphyromonas* and *Cytophaga fermentans*, were clustered in the same branch and the facultative anaerobes *Marinilabilia* and *Cytophaga fermentans* formed a subcluster in the branch of the anaerobic bacteria. Phylogenetic analysis using 16S rDNA sequences gave a similar result but with a lower bootstrap value for each cluster. The *gyrB* sequences of *Marinilabilia salmonicolor* and *Marinilabilia agarovorans* were the same, and the relatedness of their chromosomal DNA, as determined by DNA–DNA hybridization, was greater than 70%. These genetic aspects led to the conclusion that *M. salmonicolor* IFO 15948^T and *M. agarovorans* IFO 14957^T belong to a single species. Since *M. salmonicolor* was described first, as *Cytophaga salmonicolor*, *M. salmonicolor* is a senior subjective synonym of *M. agarovorans*. Therefore, the name *M. salmonicolor* should be retained and strain IFO 14957^T should be reclassified as *M. salmonicolor*. However, the agar-degrading ability of strain IFO 14957^T is a prominent biochemical characteristic. It is therefore proposed that strain IFO 14957^T should be renamed *M. salmonicolor* biovar *agarovorans*.

Keywords: *Marinilabilia*, *Cytophaga*–*Flavobacterium*–*Bacteroides* complex, *gyrB*, phylogeny

INTRODUCTION

Winogradsky (1929) established the genus *Cytophaga* for aerobic, cellulolytic soil bacteria showing gliding motility. Subsequently, this genus has included mor-

phologically similar bacteria exhibiting gliding motility. Many of them were isolated from marine environments and degraded cellulose, agar or chitin (Bachmann, 1955; Stanier, 1940, 1941, 1942; Veldkamp, 1961). Some of these species were shown to grow not only aerobically but also anaerobically (Bachmann, 1955; Veldkamp, 1961). More recent taxonomic investigations have shown an overlap between the genera *Cytophaga* and *Flavobacterium* (Callies & Mannheim, 1978; Christensen, 1977; Hayes, 1977; Oyaizu & Komagata, 1981; Shewan & McMeekin, 1983). The bacteria in these groups were then called the *Cytophaga*–*Flavobacterium* complex, and included the genera *Flexibacter*, *Microscilla*, *Sporocytophaga* and *Chitinophaga*, which resemble

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

The DDBJ accession numbers for the *gyrB* sequences reported in this paper are AB15029 (IFO 14291^T), AB15026 (ATCC 43767^T), AB15024 (IAM 14198^T), AB15025 (IFO 14944^T), AB15038 (IFO 16043^T), AB15037 (IAM 12607^T), AB15028 (IFO 14943^T), AB15032 (IFO 15948^T), AB15033 (IFO 14957^T), AB15036 (IAM 14210^T), AB15035 (IFO 12017^T), AB15027 (ATCC 43766^T), AB15030 (JCM 6326^T), AB15031 (IFO 15936^T) and AB15034 (JCM 2053^T).

Cytophaga or *Flavobacterium* in their morphological and phenotypic characteristics (Christensen, 1977; Shewan & McMeekin, 1983).

Systematic bacterial studies have advanced by adopting molecular approaches such as DNA-rRNA hybridization (Palleroni *et al.*, 1973), 16S rRNA cataloguing (Woese *et al.*, 1985) and 16S rRNA sequencing (Woese, 1987). Such investigations have revealed an unexpected relationship between the *Cytophaga-Flavobacterium* group and the genus *Bacteroides* (Gherna & Woese, 1992; Paster *et al.*, 1985; Weisburg *et al.*, 1985). The *Cytophaga-Flavobacterium-Bacteroides* (CFB) group currently represents one of eleven phyla of bacteria (Olsen & Woese, 1993; Woese, 1987). Phylogenetic studies that have used 16S rRNA sequences, chemotaxonomic studies and other phenotype analyses have resulted in several taxa of the CFB complex being rearranged (Bernardet *et al.*, 1996; Nakagawa & Yamasato, 1996; Nakagawa *et al.*, 1997; Segers *et al.*, 1993; Shah & Collins, 1988; Steyn *et al.*, 1998).

The genus *Marinilabilia* was created for the marine, facultatively anaerobic *Cytophaga* species, *Cytophaga salmonicolor* and *Cytophaga agarovorans*, by Nakagawa & Yamasato (1996). These strains were originally isolated from marine mud and were described by Veldkamp (1961). Phylogenetically, these facultatively anaerobic species in the CFB complex clustered with *Bacteroides* (Nakagawa & Yamasato, 1996; Paster *et al.*, 1994). Their phylogenetic position, facultatively anaerobic properties and chemotaxonomic characteristics prompted Nakagawa & Yamasato (1996) to transfer *Cytophaga salmonicolor* and *Cytophaga agarovorans* to a new genus, *Marinilabilia*, as *Marinilabilia salmonicolor* and *Marinilabilia agarovorans*. These two species have highly similar 16S rDNA and almost identical phenotypic characteristics (Nakagawa & Yamasato, 1996; Reichenbach, 1989; Veldkamp, 1961). However, there were significant differences between these two species in their ability to decompose agar and in the G+C contents of their chromosomal DNA: 37 mol% for *M. salmonicolor* and 41 mol% for *M. agarovorans* (Reichenbach, 1989). Nakagawa & Yamasato (1996) concluded from these points that *M. agarovorans* and *M. salmonicolor* are closely related but distinct species.

Since a large part of the 16S rRNA sequence is important for its function, the evolutionary development of 16S rDNA is very slow (Olsen & Woese, 1993). Therefore, the resolution of a molecular phylogenetic analysis based on 16S rDNA is rather low for the distinction of closely related organisms (Clayton *et al.*, 1995; Fox *et al.*, 1992). There are some reports indicating that 16S rRNA sequence analyses were inconsistent with the results of DNA-DNA hybridization (Rainey *et al.*, 1994; Stackebrandt & Goebel, 1994). Yamamoto & Harayama (1995) have recently developed a method that enables the amplification and direct sequencing of *gyrB* genes from a variety of

bacteria. The phylogenetic relationships of *Acinetobacter* strains deduced from the *GyrB* sequences were consistent with the results of DNA-DNA hybridization and of biochemical systematics (Yamamoto & Harayama, 1996).

In this study, we performed a phylogenetic analysis of bacterial strains in the CFB complex by using their *GyrB* amino acid sequences, and the results prompt us to reclassify the genus *Marinilabilia*.

METHODS

Bacterial strains and culture conditions. The following type strains of genus *Marinilabilia* and related taxa were studied: *M. salmonicolor* IFO 15948^T, *M. agarovorans* IFO 14957^T, *Bacteroides vulgatus* IFO 14291^T, *Bergeyella zoohelcum* ATCC 43767^T, *Chryseobacterium indologenes* IFO 14944^T, *Chryseobacterium meningosepticum* IAM 14198^T, *Cytophaga aurantiaca* IFO 16043^T, *Cytophaga fermentans* IFO 15936^T, *Cytophaga hutchinsonii* IAM 12607^T, *Empedobacter brevis* IFO 14943^T, *Porphyromonas asaccharolytica* JCM 6326^T, *Pedobacter heparinus* IFO 12017^T, *Sphingobacterium spiritivorum* IAM 14210^T, *Weeksella virosa* ATCC 43766^T and *Rikenella microfus* JCM 2053^T. Strictly anaerobic species were grown anaerobically with the BBL GasPack system (Becton Dickinson) in GAM broth (Nissui Pharmaceuticals) for *Bacteroides vulgatus* and *Rikenella microfus* and in JCM medium 156 for *Porphyromonas asaccharolytica*. All other species were grown aerobically. The strains of genus *Marinilabilia* and *Cytophaga fermentans* were grown in marine broth 2216 (Difco Laboratories). *Chryseobacterium meningosepticum*, *Chryseobacterium indologenes*, *Sphingobacterium spiritivorum*, *Pedobacter heparinus* and *Empedobacter brevis* were grown in L broth (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 5 g NaCl l⁻¹, pH 7.2). ATCC medium 118 was used for *Weeksella virosa*, while ATCC medium 44 was used for *Bergeyella zoohelcum* and IAMB-10 medium for *Cytophaga hutchinsonii* and *Cytophaga aurantiaca*. The temperature for cultivation was 30 °C except for *Bacteroides vulgatus*, *Rikenella microfus*, *Porphyromonas asaccharolytica*, *Bergeyella zoohelcum* and *Weeksella virosa*, which were grown at 37 °C.

PCR amplification. PCR templates were prepared from each strain according to Hiraishi *et al.* (1995). A PCR thermal cycler MP model TP3000 (Takara) was used for the PCR amplification and sequencing reactions. PCR was performed with Ampli-Taq Gold DNA polymerase (Perkin-Elmer) in a PCR buffer (Perkin-Elmer) containing each dNTP at 200 µM, 1.8 µM MgCl₂, each primer at 1 µM and 2.5 U enzyme in a total volume of 50 µl. A total of 41 amplification cycles was performed. The first three cycles used the following conditions: template DNA denaturation at 95 °C for 1 min, primer annealing at 50 °C for 1 min and primer extension at 72 °C for 1 min. The primer annealing temperature was elevated to 52 °C for the next three cycles and was further elevated to 56 °C for the subsequent 35 cycles to prevent non-specific amplification. The degenerate primers used for *gyrB* amplification were UP-21 (forward: 5'-TGTAACACGACGGCCAGTCA YGCNGGNGGNA-ARTTYGA-3') and 2Tr-SR1 (reverse: 5'-CCATAGCT-GCGTAGCATTTCATYTCNCCNARNCCYTT-3'). These primers were designed from amino acids 99-105 and 740-745 of the *Escherichia coli* DNA gyrase B subunit polypeptide (*GyrB*). The amplified products were purified by gel electro-

phoresis on 0.8% (w/v) low-melting-point agarose (Sea-Plaque GTG; FMC Bioproducts). The purified fragments were recovered from the agarose by a QIAEX II gel extraction kit (Qiagen) and used for sequencing. To determine the nucleotide sequences of both strands of each PCR product, inner degenerate primers 181r (reverse: 5'-CAGG-AAACAGCTATGACCARRTGNGTNCNC-3'), 184f (forward: 5'-GCAACGAGATCAACACTCMNGARGG-NGGNACNCA-3') and UP6r (reverse: 5'-CTGCGTT-CGTATATGAGCNCRCCTCNCRCCTC-3') were designed from consensus amino acid sequences of known GyrB polypeptides that correspond to amino acids 278–282, 276–281 and 498–503 of *E. coli* GyrB.

Sequencing. Sequencing was carried out with a Taq Dye Deoxy terminator cycle sequencing kit (ABI PRISM Cycle Sequencing FS Ready Reaction Kits; Applied Biosystems) with sequencing primers –21M13 (for UP-21-amplified fragments: 5'-TGTAACGACGGCCAGT-3'), SR1 (for 2Tr-SR1-amplified fragments: 5'-CCATAGCTGCGTAG-CATTCAT-3'), M13R (for 181r-amplified fragments: 5'-CAGGAAACAGCTATGACC-3'), 184sf (for 184f-amplified fragments: 5'-GCAACGAGATCAACACTC-3') and UP6sr (for UP-6-amplified fragments: 5'-CTGCGTTTCG-TATATGAGC-3'). The products were analysed by an ABI 373S DNA sequencer according to the manufacturer's instructions.

Phylogenetic analysis. The nucleotide sequences of *gyrB* were translated to amino acid sequences and these GyrB sequences were aligned by using CLUSTAL W software (Thompson *et al.*, 1994). Any gaps were excluded manually from the aligned sequences. An evolutionary tree was reconstructed with MORPHY version 2.3b3 software by using the maximum-likelihood method (Kishino *et al.*, 1990).

Preparation of chromosomal DNA. DNA for G+C content determination and DNA–DNA hybridization was obtained by the method described by Marmur (1961) and by Saito & Miura (1963) with the modifications described previously (Nakagawa *et al.*, 1996). Extracted chromosomal DNA was purified by using hydroxyapatite to exclude polysaccharides.

DNA base composition. DNA base composition of the *Marinilabilia* strains was determined by the method of Mesbah *et al.* (1989), using HPLC with an Asahipak GS320 column (7.6 × 250 mm; Showa Denko) and 50 mM NaH₂PO₄ (pH 5.0) as the mobile phase.

DNA–DNA hybridization. The genetic relatedness between the *Marinilabilia* strains was determined by using fluorometric DNA–DNA hybridization in microdilution wells according to the method described by Ezaki *et al.* (1989). The hybridization reaction was performed at 45 °C in 2 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) supplemented with 50% (v/v) formamide.

RESULTS AND DISCUSSION

gyrB sequences of strains of the CFB complex

Approximately 1.4 kbp-long DNA fragments were amplified by PCR from all the tested strains by using the primers described in Methods. The nucleotide sequences of the amplified fragments were determined. These sequences each had about 55% identity to the *gyrB* sequence of *E. coli* and exhibited 62–100% identity to each other. The amino acid sequences

deduced from the nucleotide sequences had the signature sequences of type II DNA topoisomerases, i.e. EGDSA and PL(R/K)G (Watt & Hickson, 1994). While the *gyrB* sequences of the two *Marinilabilia* species were identical, they were not closely related to those of the other species (65.0–78.9% identity in their amino acid sequences).

Phylogenetic structure of the CFB complex based on GyrB amino acid sequences

The phylogenetic tree (Fig. 1) for the strains of *Marinilabilia* and related bacteria was derived from their GyrB amino acid sequences by using the maximum-likelihood method. *Chryseobacterium meningosepticum*, *Chryseobacterium indologenes*, *Bergeyella zoohelcum*, *Weeksella virosa* and *Empedobacter brevis*, which each possess menaquinone-6 as the major molecule of the quinone system (Nakagawa & Yamamoto, 1996), formed one cluster. The strains of genus *Marinilabilia* were clustered with the other anaerobic species, *Bacteroides vulgatus*, *Porphyromonas asaccharolytica*, *Cytophaga fermentans* and *Rikenella microfus*. The sphingolipid-containing aerobic species (Takeuchi & Yokota, 1992; Yano *et al.*, 1982) *Pedobacter heparinus* IFO 12017^T and *Sphingobacterium spiritivorum* IAM 14210^T formed a cluster that branched earlier than the clusters just mentioned. *Cytophaga hutchinsonii*, the type species of the genus *Cytophaga*, and *Cytophaga aurantiaca* formed a deeper cluster that fell outside the sphingolipid-containing group.

Among the anaerobic species, the facultatively anaerobic species *Cytophaga fermentans* was located with the same lineage as the genus *Marinilabilia*, and their GyrB amino acid sequences were similar to each other (78.9% identity). A bootstrap analysis indicated that the anaerobic cluster (*Marinilabilia* strains, *Cytophaga fermentans*, *Rikenella microfus*, *Porphyromonas asaccharolytica* and *Bacteroides vulgatus*) had a confidence value of 96%. The lineage of the facultatively anaerobic species (genera *Marinilabilia* and *Cytophaga fermentans*) also had a high confidence value (99%).

We also reconstructed a phylogenetic tree on the basis of the *gyrB* nucleotide sequences. The constructed tree topology was almost identical to that based on GyrB amino acid sequences with some exceptions. A match between the *gyrB*- and GyrB-based trees was observed when only the first two bases of each codon were used for the analysis (data not shown).

Comparison with other phylogenetic methods

Analyses by some researchers (Nakagawa & Yamamoto, 1996; Paster *et al.*, 1994) of the phylogenetic relationships among the genus *Marinilabilia* and related bacteria have been based on 16S rDNA, and it was suggested that genus *Marinilabilia* falls within a bacteroides subcluster of the CFB phylum. Paster *et al.*

Fig. 1. Phylogenetic tree for genus *Marinilabilia* and related bacteria deduced from GyrB amino acid sequences by the maximum-likelihood method and using the JTT-F model (Jones *et al.*, 1992) for the transition matrix. The numbers shown next to the nodes indicate the percentage bootstrap probability by resampling with the estimated-likelihood method (Kishino *et al.*, 1990). Bar, 0.1 substitutions per mean amino acid residue.

(1994) have suggested that the cluster of *M. salmonicolor* and *Cytophaga fermentans* and that of *Rikenella microfus* and *Bacteroides putredinis* were connected with other bacteroides and their relatives at a deeper branch. However, the bootstrap confidence level was only 76%, so it is not clear whether these species are included in the bacteroides subcluster or form another sub-group of the CFB phylum. Nakagawa & Yamasato (1996) performed a phylogenetic analysis of the CFB phylum by using 16S rDNA sequences. They showed a high level of 16S rDNA sequence identity (98.9%) for the two *Marinilabilia* species. They also reported that genus *Marinilabilia* clustered with *Bacteroides splanchnicus* rather than with *Cytophaga fermentans*. However, the bootstrap confidence level for this clustering was not very high.

In contrast to the previous observations, the bootstrap confidence level of the anaerobic cluster in the phylogenetic tree based on the GyrB amino acid sequences was quite high (96%). This high confidence level prompts us to conclude that genus *Marinilabilia* is included in the bacteroides subcluster of the CFB phylum.

DNA–DNA hybridization of genus *Marinilabilia*

The close relationship between *M. salmonicolor* and *M. agarovorans* has been indicated from phylogenetic analyses of 16S rDNA sequences (Nakagawa & Yamasato, 1996) and GyrB sequences (this study). To confirm this close relationship, a DNA–DNA hybridization experiment was performed. The DNA relatedness between these two strains in genus *Marinilabilia* is consistent with the results of the GyrB sequence analysis (Table 1). The DNA relatedness between *M.*

salmonicolor IFO 15948^T and *M. agarovorans* IFO 14957^T was over 70%. However, *Cytophaga fermentans* IFO 15936^T showed only a low level of DNA relatedness to each of the *Marinilabilia* species.

Re-evaluation of the species of genus *Marinilabilia*

The close relationship between *M. salmonicolor* and *M. agarovorans* has been suggested previously on the basis of similarities in the biochemical and chemotaxonomical characteristics of these organisms (Hamana *et al.*, 1995; Nakagawa & Yamasato, 1996; Reichenbach, 1989). The obvious differences were the ability to decompose agar and the G+C content of DNA. Reichenbach (1989) has mentioned that there was a small percentage difference in the G+C content between *M. agarovorans* and *M. salmonicolor*. The G+C contents of *M. agarovorans* and *M. salmonicolor* were reported as 41 and 37%. We determined the G+C content of DNA of these bacteria by an HPLC analysis of the deoxynucleosides that were generated by P1 nuclease digestion and bacterial alkaline phosphatase dephosphorylation of DNA. Our results show that the G+C contents of these two bacteria were almost the same. The G+C contents of strains IFO 14957^T (*M. agarovorans*) and IFO 15948^T (*M. salmonicolor*) were 41.2 and 41.5%. This information enables us to conclude that *M. salmonicolor* IFO 15948^T and *M. agarovorans* IFO 14957^T belong to a single species. Since *M. salmonicolor* was described first, as *Cytophaga salmonicolor*, *M. salmonicolor* is a senior subjective synonym of *M. agarovorans*. Strain IFO 14957^T should be reclassified as *M. salmonicolor*. However, the agar-degrading ability of strain IFO 14957^T is a prominent biochemical characteristic;

Table 1. DNA base composition and DNA–DNA hybridization results for *Marinilabilia* strains

Strain	G + C content (mol %)	Reassociation (%) with DNA from:	
		IFO 14957 ^T	IFO 15948 ^T
<i>Marinilabilia agarovorans</i> IFO 14957 ^T	41.2	100	78
<i>Marinilabilia salmonicolor</i> IFO 15948 ^T	41.5	88	100
<i>Cytophaga fermentans</i> IFO 15936 ^T	ND	5	5

ND, Not determined.

therefore this strain should be recognized as biovar *agarovorans* of *M. salmonicolor*.

Emended description of *Marinilabilia salmonicolor* (Veldkamp 1961) Nakagawa and Yamasato 1996

Marinilabilia salmonicolor (sal.mo.ni'co.lor. L. masc. gen. n. *salmo* of salmon; L. masc. n. *color* colour; M. L. adj. *salmonicolor* salmon-coloured).

Cells are slender, flexible, cylindrical rods with rounded or slightly tapering ends, 0.3–0.5 × 2–50 µm (usually around 10–20 µm in length). Resting stages are not observed. Motile by gliding. Gram-negative. Colour of cell mass is salmon to pink. Chemo-organotroph. Facultative anaerobe. Good growth is obtained in a normal aerobic atmosphere. Marine organism that requires elevated salt concentrations. Peptones, Casamino acids, yeast extracts, ammonium and nitrate are suitable nitrogen sources. Arabinose, xylose, glucose, galactose, mannose, fructose, sucrose, lactose, maltose, cellobiose, trehalose, raffinose, inulin and starch are fermented. Gelatin is slowly liquefied. Catalase is produced. The optimum temperature is 28–37 °C. The optimum pH is around 7. The quinone is MK-7. The major polyamine is spermidine. The G + C content of the DNA is 41.2–41.5 mol%. Type strain is IFO 15948^T. The other strain, IFO 14957, which can degrade agar, is a reference strain of biovar *agarovorans*.

Concluding remarks

The description of genus *Marinilabilia* should also be emended. The G + C content of this genus is 41.2–41.5 mol% and there is only one species included. Other characteristics of this taxon are the same as those described for the genus and those described by Nakagawa & Yamasato (1996).

The another facultatively anaerobic species, *Cytophaga fermentans*, is clearly misclassified in the genus *Cytophaga*. This species is distantly related to other *Cytophaga* species on the basis of 16S rDNA sequence comparisons (Nakagawa & Yamasato, 1993, 1996; Paster *et al.*, 1994) and GyrB sequence comparisons. *Cytophaga fermentans* is a neighbour of genus *Marinilabilia*. However, there is not a close identity (78.9%)

between their GyrB sequences, and DNA relatedness between *Cytophaga fermentans* and *Marinilabilia* strains was very low (Table 1). Therefore, a new genus should be created to accommodate *Cytophaga fermentans*.

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