

## *Sufflavibacter maritimus* gen. nov., sp. nov., Novel *Flavobacteriaceae* Bacteria Isolated from Marine Environments

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**Abstract** Four Gram-negative, chemoheterotrophic, non-motile, yellow-colored strains were isolated from the East Sea or from deep-sea sediments of Nankai Trough by standard dilution plating. Characterization by polyphasic approaches indicated that the four strains are members of the same species. Phylogenetic analyses based on 16S rRNA gene sequences revealed that the strains formed a coherent and novel genus-level lineage within the family *Flavobacteriaceae*. The dominant cellular fatty acids were i-C15:0, 3-OH i-C17:0, and 2-OH i-C15:0 and/or C16:1 ω7c. Predominance of 2-OH i-C15:0 and/or C16:1 ω7c clearly differentiated the strains from closely related members. The DNA G+C contents ranged 35.1–36.2 mol%. It is proposed, from the polyphasic evidence, that the strains should be placed into a novel genus and species named *Sufflavibacter maritimus* gen. nov., sp. nov., with strain IMCC1001<sup>T</sup> (=KCCM 42359<sup>T</sup>=NBRC 102039<sup>T</sup>) as the type strain.

**Keywords:** East Sea, Nankai Trough, polyphasic taxonomy, *Sufflavibacter maritimus*

The family *Flavobacteriaceae* [3, 36] is one of the major phylogenetic lineages within the phylum *Bacteroidetes* [14], which generally comprise 6–30% of total bacterial communities in sea water, as determined by fluorescence *in-situ* hybridization [9, 10, 16]. The members of the *Bacteroidetes* comprise 11–22% in tidal flat sediments [22, 38] or near-shore sediment [26], but much lower portions in terrestrial environments, solar saltern, or deep-sea sediment samples [6, 18, 19, 23, 34, 39]. The family *Flavobacteriaceae* has been considered to have positive correlation with the organic matters and play an important role for the degradation of polymeric substances derived from algal biomass [2].

Members of the family are characterized as chemoorganotrophic, nonspore-forming, Gram-negative, and nonmotile or motile by gliding. The majority members of the family contain carotenoids and/or flexirubin-type pigments. Menaquinone 6 serves as their only or major respiratory quinone. Their major cellular fatty acids are branched or hydroxy fatty acids, with high levels of i-C15:0 and 3-OH i-C17:0 [3]. Most of the members have been isolated from marine environments including seawater, surface of macro alga, animals, sediments, *etc.* [38]. Recently, the number of genera included within the family increased rapidly because of many novel isolates from diverse marine environments. In the present study, we propose four novel isolates (IMCC1001<sup>T</sup>, NHD065, NHD150, and NHD261) utilizing a broad range of carbon sources, originated from two different marine environments, as a novel genus and species, based on the findings that the strains formed a distinct phylogenetic clade in the family *Flavobacteriaceae*, and they were phenotypically different from other related genera in the family. The strains were collected during the acquisition of valuable bioresources from a variety of marine environments. Its versatile carbohydrates metabolism has possibilities for biotechnological application.

Strain IMCC1001<sup>T</sup> was isolated from seawater near Goseong, Korea, by the serial dilution and plating method on marine agar 2216 (MA; Difco). Strains NHD065, NHD150, and NHD261 were isolated from deep-sea sediments at Nankai Trough, Japan. These isolates were further routinely cultured on MA at 30°C for polyphasic taxonomy.

The physiological and morphological characterization was conducted according to the procedures described in Bae *et al.* [1] and Cho and Giovannoni [4]. The potential of the strains to metabolize 95 different carbon sources was examined by using GN2 Microplates (Biolog). Biochemical tests were carried out using API 20NE and API ZYM

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**Table 1.** Phenotypic characteristics of *Sufflavibacter maritimus* and closely relative genera in the family *Flavobacteriaceae*.

	1 <sup>b</sup> (n=4)	2 <sup>b</sup> (n=5)	3 <sup>b</sup> (n=2)	4 <sup>b</sup> (n=2)
Gliding motility	-	v	v	+
Requirement of Na <sup>+</sup> for growth	v (-)	v	+	+
Acid formation from glucose	+	v	v	+
Growth range				
Temperature <sup>a</sup>	3–42 (30–34)	0–41 (22–32)	4–39 (21–30)	4–37 (23–30)
Salinity <sup>a</sup>	0–15 (2.0–5.0)	0–20 (2–5)	1–15 (3–4)	1–15
pH <sup>a</sup>	4–12 (7–8)	5–10 (7.5–8.5)	6.0–9.5 (7.5)	6–10 (7–8)
Hydrolysis of				
Agar	-	v	-	-
Casein	v (+) <sup>d</sup>	v	v	v
Cellulose (CM-cellulose filter paper)	ND	-	-	ND(-) <sup>c</sup>
Elastin	ND	+	ND	ND
Starch	+	+	-	+
DNA	+	v	-	v
Urea	-	v	-	-
Alginic acid	ND	+	ND(-) <sup>c</sup>	ND
Tween 20	ND	+	+	v
Tween 40	v (+) <sup>d</sup>	+	v	+
Tween 80	v (+) <sup>d</sup>	+	v	+
Nitrate reduction	v (-) <sup>d</sup>	v	-	-
Production of				
H <sub>2</sub> S	-	v	v	-
Indole	v (-) <sup>d</sup>	-	-	-
Acetoin	+	-	-	v
DNA G+C content (mol%)	35.1–36.2	36.7–40.4	32.7–36.1	39.6–39.9

1, *Sufflavibacter maritimus* (present study); 2, *Salegentibacter* [20, 27, 29, 31, 32]; 3, *Mesonnia* [28, 33]; 4, *Gramella* [25, 30]. All strains were positive for hydrolysis of gelatin, oxidase, and catalase. All strains were negative for hydrolysis of chitin and production of Flexirubin pigment. Abbreviation: +, positive; -, negative; v, variable; ND, not detected.

<sup>a</sup>Optimum range shown in parenthesis.

<sup>b</sup>Characteristics for all strains or species are presented.

<sup>c</sup>Only one species was tested for this character in the genus and showed negative result.

<sup>d</sup>In case of variable results for all strains, results of the type strain are presented in the parenthesis.

strips (bioMérieux). The physiological, biochemical, and morphological characteristics of the strains studied are given below in the species description and in Table 1. Briefly, the isolates were facultatively anaerobic, nonmotile, chemoheterotrophic, rod-shaped bacteria that utilized a wide range of carbon sources, including polymeric substances, sugar acids, amino acids, and nucleosides.

Susceptibility of strain IMCC1001<sup>T</sup> to antimicrobial agents was tested for ampicillin (10 µg), chloramphenicol (25 µg), erythromycin (15 µg), kanamycin (30 µg), penicillin G (10 µg), rifampicin (50 µg), streptomycin (10 µg), tetracycline (30 µg), gentamicin (10 µg), and vancomycin (30 µg) according to the procedure described in Cho and Giovannoni [5].

To determine whole-cell fatty acid content, all the strains were grown on MA or TSI agar (Difco) at 30°C for 24 h. Fatty acid methyl esters were prepared and analyzed

according to the standard protocol of the Microbial Identification System (MIDI). The dominant fatty acids were iso-C15:0, iso-C17:0 3-OH, and 2-OH i-C15:0 and/or C16:1 $\omega$ 7c (summed feature 3) (Table 2). Relatively high amounts of summed feature 3 discriminated the strains from closely related members of the family *Flavobacteriaceae*.

Genomic DNA extraction and 16S rRNA gene sequencing were conducted according to previously described procedures [24]. Nearly complete 16S rRNA sequences (1,459–1,491 bp) were determined and compared with the sequences held in the GenBank database. The GenBank accession numbers for the 16S rRNA gene sequence of strains IMCC1001<sup>T</sup>, NHD065, NHD150, and NHD261 are DQ868538, DQ993337, DQ993339, and DQ993340, respectively. The 16S rRNA gene sequence of strain IMCC1001<sup>T</sup> had only 1 base mismatch to the three marine sediment isolates, suggesting that they are the same species.

**Table 2.** Cellular fatty acid compositions of the *Sufflavibacter maritimus* and closely related genera in the family *Flavobacteriaceae*.

Fatty acid	1	2 (n=3)	3	4	5
<b>Straight-chain fatty acids</b>					
C15:0	4.2	6.3–6.6	7.6	4.8	7.1
C16:0	1.5	1.0–1.1			
<b>Branched fatty acids</b>					
i-C14:0					1.4
i-C15:0	16.3	21.6–22.3	8.5	21.2	14.4
a-C15:0	4.7	1.5–1.5	8.5	4.2	7.6
i-C15:1	4.8	6.9–7.1	17.7	7.9	1.2
a-C15:1			3.5	0.4	
i-C16:0		1.3	3.1	6.3	13.1
i-C16:1			2.6	4.2	5.8
i-C17:1 $\omega$ 9	1.4	8.5–8.9	2.0	5.1	3.5
a-C17:1 $\omega$ 9				1.9	2.0
<b>Unsaturated fatty acids</b>					
C15:1 $\omega$ 6c		1.7–2.2	5.7	1.8	1.9
C17:1 $\omega$ 6c		1.1–1.2	4.1	2.4	3.6
<b>Hydroxy fatty acids</b>					
C15:0 2-OH	2.5		2.4	1.4	2.0
C15:0 3-OH	1.7	1.6–1.7	3.9		
i-C15:0 3-OH	6.3	4.4–4.7	2.5	3.2	1.3
i-C16:0 3-OH	2.1	1.3–1.5	5.9	6.0	5.9
C17:0 2-OH			4.7	1.8	2.6
i-C17:0 3-OH	18.4	16.4–16.5	5.9	14.5	6.7
<b>Summed feature 3<sup>a</sup></b>	25.0	16.9–18.0	6.1	5.1	11.4
<b>Unknown</b>	4.5	1.1–1.4		3.3	4.6

Species: 1, *Sufflavibacter maritimus* IMCC 1001<sup>T</sup> on Marine agar 2216 (present study); 2, *Sufflavibacter maritimus* NHD strains on TSI medium (present study); 3, *Salegentibacter salegens* DSM5424<sup>T</sup> [27]; 4, *Mesonina algae* KMM 3909<sup>T</sup> [28]; 5, *Gramella echinicola* KMM 6050<sup>T</sup> [30].

\*Fatty acids displaying lower than one percent proportion are omitted.

<sup>a</sup>Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3, iso-C15:0 2-OH and/or C16:1 $\omega$ 7c.

The strains showed the highest sequence similarities with the genera *Salegentibacter* (91.8–92.9% similarity) and *Mesonina* (91.9–93.0% similarity). Thereafter, the 16S rRNA gene sequences of the strains were aligned with validly reported members of the genera *Salegentibacter*, *Mesonina*, *Gramella*, *Gillisia*, and *Psychroflexus* using the PHYDIT program [7] (available at <http://plaza.snu.ac.kr/~jchun>). Approximately 1,370 nucleotide positions, ranging from *Escherichia coli* equivalent positions between 28 and 1,475, were aligned based on the 16S rRNA secondary structure of *E. coli* [17]. Phylogenetic trees were constructed on the basis of neighbor-joining [37] with Jukes-Cantor distance [21], maximum likelihood, and maximum parsimony algorithms (Fig. 1). The tree topology of resultant neighbor-joining and maximum parsimony trees was evaluated by bootstrap analysis [12] using 1,000 replicates. Phylogenetic analyses revealed that the strains formed an independent

phyletic lineage within the family *Flavobacteriaceae*. The strains shared a supra-generic phyletic lineage with the genera *Gillisia*, *Gramella*, *Mesonina*, *Psychroflexus*, and *Salegentibacter*; however, the phylogenetic position in this clade was not strongly supported by bootstrap analysis (Fig. 1).

The DNA G+C content was 35.1–36.2 mol%, as determined by HPLC using a symmetry reversed-phase C<sub>18</sub> column (Waters) [40]. The major respiratory quinone was determined to be MK-6 by HPLC analysis according to Collins [8]. Using the bathochromic shift test with 20% (w/v) KOH [11], no flexirubin-type pigments were detected in any of the strains.

Taxonomic evidence collected in this study clearly demonstrated that strains IMCC1001<sup>T</sup>, NHD065, NHD150, and NHD261 should be classified as a novel genus and species in the family *Flavobacteriaceae*, for which the name *Sufflavibacter maritimus* gen. nov., sp. nov. is proposed.

Bacterial phylotypes related to this novel genus were reported from sediments of the North-West area of the Pacific Ocean (DQ855467, unpublished, from GenBank description), deep-sea corals [35], and seawater of the Mediterranean Sea [13, 15]. This finding implies that the phylogenetic group containing our novel strains and several related phylotypes might be a cosmopolitan lineage in the marine environments. Further study will be required to elucidate the abundance and ecological role of the phylogenetic group in diverse marine environments.

#### Description of *Sufflavibacter* gen. nov.

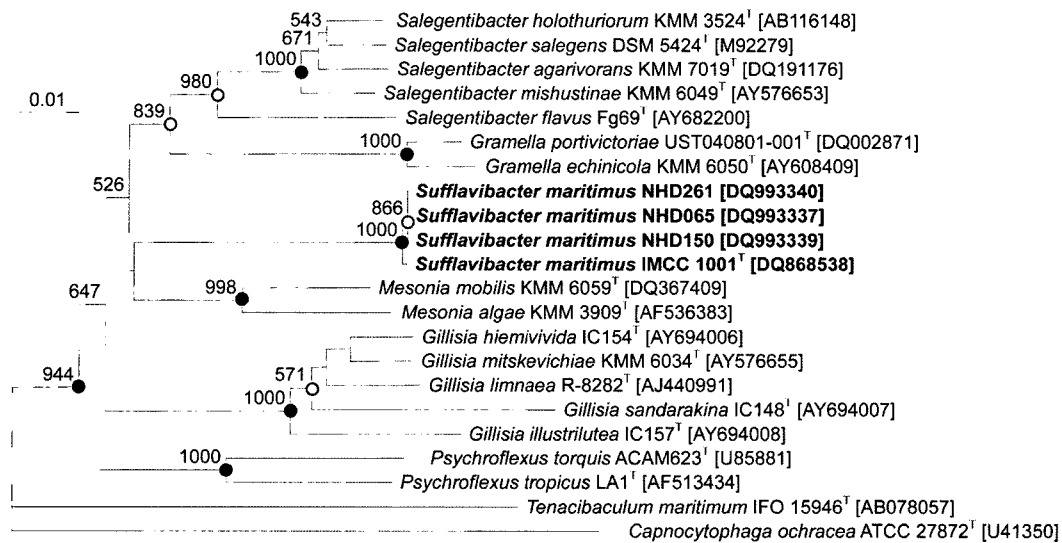
*Sufflavibacter* (Suf.fl.a.vi.bac'ter. L. adj. *sufflavus* yellowish; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Sufflavibacter* a yellowish rod).

Cells are facultatively anaerobic, Gram negative, non-motile rods. Yellow colonies are formed on MA. Flexirubin-type pigments are not detected. DNA G+C content is 35.1–36.2 mol%. Major respiratory quinone is MK-6. The major cellular fatty acids are i-C15:0, i-C17:0 3-OH, and C16:1 $\omega$ 7c and/or 2-OH i-C15:0 (summed feature 3). Oxidase- and catalase-positive. As determined by 16S rRNA gene sequence analysis, the genus *Sufflavibacter* is a member of the family *Flavobacteriaceae* within the phylum *Bacteroidetes*. The type species is *Sufflavibacter maritimus*.

#### Description of *Sufflavibacter maritimus* sp. nov.

*Sufflavibacter maritimus* (ma.ri'ti.mus. L. masc. adj. *maritimus* maritime, marine).

In addition to the description of the genus, the species displays the following characteristics. Cells are long rods, 0.4–0.8  $\mu$ m wide and 0.9–1.8  $\mu$ m long. Growth is detected at 3–42°C (optimum at 30–34°C). The pH range for growth is 4–12 (optimum at pH 7–8). Growth occurs at 0–15% NaCl (optimum at 2.0–2.5% for IMCC 1001<sup>T</sup> and



**Fig. 1.** Phylogenetic tree based on nearly complete 16S rRNA gene sequences (positions 28 to 1,475 of *E. coli* numbering system) showing the relationships between strains IMCC1001<sup>T</sup>, NHD065, NHD150, and NHD261 and closely related genera of the family *Flavobacteriaceae*. The tree is based on the Jukes and Cantor distances model and the neighbor-joining algorithm. Bootstrap values over 50% for 1,000 resamples are shown. Nodes recovered by the maximum likelihood algorithm and parsimony method are represented as filled circle (>70%) and hollow circle (>50%) at each node. Scale bar, 0.01 substitutions per nucleotide position.

3.5–5.0 for NHD strains). Urease activity is negative. Nitrate reduction, indole production, and casein degradation showed variable results. Starch, gelatin, esculin, and DNA are degraded but agar and chitin are not. H<sub>2</sub>S is not produced. O-F test of glucose is fermentative. Acid is produced from glucose. Depending on the API ZYM test, alkaline phosphatase, esterase (C4), esterase-lipase (C8), valine arylamidase, cystine arylamidase, trypsin,  $\alpha$ -chymotrypsin, acid phosphatase,  $\alpha$ - and  $\beta$ -galactosidases,  $\beta$ -glucuronidase, N-acetyl- $\beta$ -glucosaminidase, and  $\alpha$ -mannosidase activities are present, and naphthol-AS-BI-phosphohydrolase and leucine arylamidase activities are weak. Utilization by all strains (Biolog GN2 microplate data) is observed for  $\alpha$ -cyclodextrin, dextrin, glycogen, D-fructose, D-galactose, gentiobiose,  $\alpha$ -D-glucose,  $\alpha$ -D-lactose, lactulose, maltose, D-mannose, D-melibiose,  $\beta$ -methyl-D-glucoside, D-raffinose, sucrose, D-trehalose, turanose, D-galacturonic acid, D-glucuronic acid,  $\alpha$ -ketoglutaric acid, bromo succinic acid, succinamic acid, L-alanyl-glycine, L-asparagine, L-glutamic acid, glycyl-L-aspartic acid, L-ornithine, L-proline, glycerol, and D,L-a-glycero phosphate. Tweens 40 and 80, adonitol, L-arabinose, cellobiose, D-psicose, L-rhamnose, methyl pyruvate, mono-methyl succinate, D-galacturonic acid lactone, D-gluconic acid, D-glucosaminic acid,  $\gamma$ -hydroxy butyric acid,  $\alpha$ -keto-butyric acid,  $\alpha$ -ketovaleric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, L-aspartic acid, glycyl-L-glutamic acid, L-leucine, L-phenylalanine, L-pyroglutamic acid, D- and L-serine, L-threonine, D,L-carnitine,  $\gamma$ -aminobutyric acid, putrescine,

2-amino-ethanol, glucose-1-phosphate, and glucose-6-phosphate are utilized by two or three strains. N-Acetyl-D-galactosamine, acetic acid, glucuronamide, alaninamide, L-alanine, hydroxyl-L-proline, inosine, and uridine were utilized by only one strain. The major cellular fatty acids are i-C17:0 3-OH, i-C15:0, summed feature 3, i-C15:0 3-OH, i-C15:1, a-C15:0, and C15:0. The type strain is susceptible to chloramphenicol (25  $\mu$ g), erythromycin (15  $\mu$ g), penicillin G (10  $\mu$ g), rifampicin (50  $\mu$ g), and tetracycline (30  $\mu$ g). The type strain is IMCC1001<sup>T</sup> (=KCCM 42359<sup>T</sup>=NBRC 102039<sup>T</sup>), isolated from seawater.

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