Vitellibacter vladivostokensis gen. nov., sp. nov., a new member of the phylum *Cytophaga–Flavobacterium–Bacteroides*

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A novel heterotrophic, yellow-orange-pigmented, non-motile, asporogenic, strictly aerobic, Gram-negative, oxidase and catalase-positive bacterium KMM 3516^T was isolated from the holothurian *Apostichopus japonicus* collected from Troitsa Bay in the Gulf of Peter the Great (Sea of Japan) during November 1997. 16S rDNA sequence analysis revealed that strain KMM 3516^T was a member of the family *Flavobacteriaceae*. The DNA G + C content of KMM 3516^T was 41.3 mol%. Major respiratory quinone was MK-6. Predominant fatty acids were i15:0 and α 15:0 (68.8 and 8.4 %, respectively). On the basis of phenotypic, chemotaxonomic, genotypic and phylogenetic characteristics, the novel bacterium has been designated *Vitellibacter vladivostokensis* gen. nov., sp. nov. The type strain is KMM 3516^T (=NBRC 16718^T).

Bacteria belonging to the phylum *Cytophaga–Flavobacterium–Bacteroides* (CFB) are common inhabitants of marine environments. These bacteria are heterotrophic, usually pigmented, motile by gliding or non-motile micro-organisms, possess highly active enzymes and occupy different ecological niches (Reichenbach, 1992; Barbeyron *et al.*, 2000; Humphry *et al.*, 2001; Politz *et al.*, 2000). Members of genera such as *Cellulophaga*, *Tenacibaculum* and *Flavobacterium* may be free-living or host-associated organisms (Johansen *et al.*, 1999; Suzuki *et al.*, 2001; McCammon & Bowman, 2000). Recently, we reported the isolation of a new representative of the CFB phylum, *Arenibacter latericius* gen. nov., sp. nov. (Ivanova *et al.*, 2001), strains of which were found in bottom sediment samples collected in the South China Sea, on the fronds of the brown alga grown in the Kuril Isles region (Sea of Okhotsk) and in the edible holothurian *Apostichopus japonicus*, which inhabits the coastal waters of the Sea of Japan. According to phylogenetic analysis, the genus *Arenibacter* forms single cluster with representatives of the recently described genera *Muricauda* and *Zobellia* (Bruns *et al.*, 2001; Barbeyron *et al.*, 2001). The latter accommodates two species, *Zobellia uliginosa* (formerly *Cellulophaga*

uliginosa; Bowman, 2000) and *Zobellia galactanivorans* (Barbeyron *et al.*, 2001). In the present work, we examined by polyphasic taxonomy another environmental isolate associated with the holothurian *Apostichopus japonicus*, and found a novel bacterium belonging to the CFB phylum and closely related to the *Arenibacter–Zobellia–Muricauda* phylogenetic cluster. Based on polyphasic taxonomy data (phylogenetic, pheno- and genotypic analyses, menaquinone and cellular fatty acid compositions) obtained and presented in this work, we describe a new member of the family *Flavobacteriaceae*, *Vitellibacter vladivostokensis* gen. nov., sp. nov.

Strain KMM 3516^T was isolated from gut of the holothurian *Apostichopus japonicus* collected in Troitsa Bay, in the Gulf of Peter the Great (Sea of Japan) in November 1997 from a depth of 8 m (salinity 33 ‰, temperature 12 °C). For strain isolation 0.1 ml tissue homogenates was transferred on to plates of Marine agar 2216 (Difco). After primary isolation and purification strain KMM 3516^T was cultivated at 28 °C on the same medium and stored at –80 °C in Marine broth (Difco) supplemented with 20 % (v/v) glycerol.

Oxidative or fermentative utilization of glucose was determined on the Hugh & Leifson medium modified for marine bacteria (Lemos *et al.*, 1985). Catalase activity was tested by addition of 3 % (v/v) H₂O₂ solution to a bacterial colony and observed for the appearance of gas. Flexirubin

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

The GenBank accession number for the 16S rDNA sequence of *Vitellibacter vladivostokensis* KMM 3516^T is AB071382.

pigments were examined by Fautz & Reichenbach (1980) method. Gram-staining, degradation of alginic acids (1 %, w/v) and agar (1.5 %, w/v), oxidase and alkaline phosphatase activity, production of acid from carbohydrates, hydrolysis of starch, casein, gelatin, cellulose (filter paper), Tween 20, Tween 40, Tween 80, DNA and urea, nitrate reduction, production of hydrogen sulphide and indole were carried out according to the methods of Smibert & Krieg (1994). Hydrolyses of chitin (1 %, w/v), elastin (2 %, w/v) and CM-cellulose (1 %, w/v) were tested by appearance of clear zones around colonies. A medium containing 0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g Yeast Extract (Difco) and 0.4 % (w/v) carbon source in 1 l artificial sea water was used to examine carbon-source utilization. Carbon sources tested were arabinose, glucose, lactose, mannose, sucrose, inositol, sorbitol, mannitol, glycerol, *N*-acetylglucosamine, acetate, citrate, malonate, tartrate and alanine. In order to study of the temperature range, bacteria were cultivated on medium A, which contained (l⁻¹) 5 g Bacto peptone (Difco), 2 g Bacto yeast extract (Difco), 1 g glucose, 0.02 g KH₂PO₄ and 0.05 g MgSO₄·7H₂O in 50 % (v/v) natural sea water and 50 % (v/v) distilled water. Bacterial growth at the different concentrations of NaCl was checked on the medium A prepared with distilled water and containing 0, 1, 2, 3, 5, 6, 8, 10 and 12 % (w/v) of NaCl. Spreading growth was observed by cultivation on the medium B, which contained (l⁻¹) 1 g Bacto peptone, 1 g yeast extract, 15 g agar and half-strength natural sea water under high moisture conditions. Gliding motility was determined as described by Bowman (2000). The cell movement at the edges of colony was verified by using phase-contrast microscopy. Susceptibility to antibiotics was examined by the routine diffusion plate method. Disks were impregnated with the following antibiotics: ampicillin (10 µg), benzylpenicillin (10 µg), carbenicillin (100 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (15 µg), neomycin (30 µg), oleandomycin (15 µg), polymyxin B (300 U), streptomycin (10 µg), tetracycline (30 µg).

To determine fatty acid composition, strains were cultivated at 28 °C for 72 h on medium A. The analysis of fatty acid methyl esters was performed by GLC [30 m × 0.25 mm Supelcowax 10 column, 205 °C] as described by Svetashev *et al.* (1995). Isoprenoid quinones were extracted and analysed by the method of Nakagawa & Yamasato (1993).

DNA was isolated following the method of Marmur (1961) and the DNA G + C content was determined by the thermal denaturation method (Marmur & Doty, 1962). The 16S rDNA gene sequence of KMM 3516^T was determined by PCR amplification and direct sequencing (Hiraishi, 1992). Conditions and reagents used for PCR amplification and sequencing of 16S rDNA were described previously (Suzuki *et al.*, 2001). The determined sequence was aligned to the alignment based on secondary structure model which is maintained by the SSU rRNA database (Van de Peer *et al.*, 2000) using the profile alignment program of CLUSTAL W

software (Thomson *et al.*, 1994). The evolutionary distances were then computed with the DNADIST program in the PHYLIP 3.572 package (Felsenstein, 1995) with the Kimura 2-parameter model (Kimura, 1980), and the phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987). To evaluate the phylogenetic trees, a bootstrap analysis with 1000 sample replications was performed with the SEQBOOT and CONSENSE programs in the PHYLIP 3.572 package. The phylogenetic position of KMM 3516^T was also verified by maximum-parsimony analysis using the PAUP version 3.1.1 program and by maximum-likelihood analysis using DNAML program in the PHYLIP 3.572 package.

Strain KMM 3516^T was Gram-negative, chemo-organotrophic with respiratory type of metabolism, non-motile by gliding, asporogenic and consisted of single flexible rods, 0.3–0.5 µm in diameter and 3–10 µm long. On marine agar, colonies were round, 2–4 mm in diameter, yellow-orange-pigmented, shiny and convex, with entire margins. It was oxidase-, catalase- and alkaline phosphatase-positive, and required Na⁺ ions for growth. Growth occurred on media containing 1–6 % (w/v) NaCl (optimum 2 %). The temperature range for growth was 4–43 °C (optimum 28 °C). The pH range of growth was 5.5–10.0 (optimum 7.5–8.5). Flexirubin pigments were formed. Gelatin, casein, elastin, Tween 20, Tween 40 and DNA were degraded. Cellulose (CM-cellulose and filter paper), chitin, agar, starch, alginate, urea, Tween 60 and Tween 80 were not hydrolysed. H₂S and indole were not produced. No acid was formed from arabinose, cellobiose, fucose, galactose, glucose, lactose, maltose, melibiose, raffinose, rhamnose, sorbose, sucrose, xylose, adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, *N*-acetylglucosamine or citrate. Glycerol, *N*-acetylglucosamine, acetate, citrate, malonate, tartrate and alanine were utilized, whereas arabinose, glucose, lactose, mannose, sucrose, inositol, mannitol and sorbitol were not. Nitrate reduction was negative. Strain KMM 3516^T was susceptible to carbenicillin, oleandomycin and lincomycin, but not to ampicillin, benzylpenicillin, streptomycin, gentamicin, neomycin, polymyxin B or tetracycline.

The whole-cell fatty acid composition of KMM 3516^T is shown in Table 1. The predominant cellular fatty acids were branched-chain saturated, namely i15:0 (68.8 %) and α15:0 (8.4 %) fatty acids. The major isoprenoid quinone was menaquinone MK-6. The G + C content of the genomic DNA of strain KMM 3516^T was 41.3 mol%.

In order to clarify the phylogenetic position of strain KMM 3516^T, the almost complete 16S rDNA sequence was determined. From the results of sequence comparison with previously described member of the CFB phylum, the highest sequence similarities were with *Zobellia* species and *Arenibacter latericius* (90.0–90.1 %). The 16S rDNA sequence similarities with other members of the family *Flavobacteriaceae* were only 86.6–90.1 %. Neighbour-joining analysis revealed that KMM 3516^T is distantly related to the genera *Zobellia* and *Arenibacter* (Fig. 1). Also

Table 1. Whole-cell fatty acid profiles (percentage content) of *V. vladivostokensis* KMM 3516^T and relatives in the CFB phylum

Strains: 1, *Vitellibacter vladivostokensis* KMM 3516^T; 2, *Arenibacter latericius* KMM 426^T; 3, *Zobellia uliginosa* CIP 104808^T; 4, *Salegentibacter salegens* DSM 5424^T; 5, *Cellulophaga lytica* ATCC 23178^T; 6, *Tenacibaculum mesophilum* MBIC 1140^T; 7, *Flavobacterium flevense* CIP 104740^T.

Fatty acid	1	2	3	4	5	6	7
i13:0					0.1	0.6	1.6
i13:0	0.5		0.3	0.1	2.2	1.1	
i14:0		0.3				0.2	0.1
i14:1	0.2	0.1				0.1	
14:0	0.1	1.1	0.9	0.4	1.3	0.1	0.6
i15:0	68.8	16.0	31.2	23.9	39.1	19.6	7.3
α15:0	8.4	13.0	3.7	5.8	4.7		3.9
i15:1	2.4	12.9	4.3	0.4	9.5	11.4	1.9
15:0	3.7	12.3	15.6	2.4	10.3	2.0	12.5
15:1	0.1	1.7	0.2	1.8	0.6	0.9	12.1
i16:0	3.0	1.4	0.3	2.1	2.7	0.4	0.8
i16:1	0.3	0.3	5.1	1.3	0.3	0.3	2.4
16:0	2.2	8.6			3.9		
16:1ω7	0.1	12.4	10.1	6.0	4.9	0.2	14.3
16:1	0.1	0.3	0.1	0.3	1.1	0.7	0.3
i17:0	2.7	1.5	0.7	0.4	3.8		0.1
α17:0	0.1	1.2	0.3	0.7			0.2
i17:1	4.0	2.7	3.5	5.6		0.4	0.7
α17:1		0.4	0.5	3.9		0.5	0.3
17:0	0.2	0.2				0.5	1.9
17:1		1.2	1.0	2.1		0.1	8.2
18:0		0.4	0.3				
18:1ω9		0.5	0.2				
i15:0 2-OH	0.6	1.5	2.1	29.3	0.4	1.0	0.9
14:0 3-OH							1.0
15:0 2-OH			0.2	0.1		0.3	
i15:0 3-OH	1.6	5.3	7.6	10.4	3.3	7.7	4.7
15:0 3-OH						2.0	1.0
i16:0 3-OH		1.8	1.1	1.3	1.6	1.1	
16:0 3-OH			2.4	1.7	0.6	3.6	1.5
i17:0 3-OH	0.8	3.4			5.4		4.4

the bootstrap probability of this lineage was low, but the clustering of KMM 3516^T and genera *Arenibacter* and *Zobellia* was shown in other analyses using maximum-parsimony and maximum-likelihood methods. In these analysis the genus *Cellulophaga* was always monophyletic and the nearest genus to the lineage of KMM 3516^T – genera *Arenibacter* and *Zobellia*. It is evident from the tree that KMM 3516^T represents a distinct sublineage within the family *Flavobacteriaceae*.

From the results of the polyphasic taxonomic analysis, it can be concluded that strain KMM 3516^T is related to currently described taxa of the phylum CFB. Phylogenetic analysis revealed that KMM 3516^T belongs to the members of the family *Flavobacteriaceae* (Fig. 1) and forms a distinct

lineage within cluster represented by *Arenibacter latericius*, *Zobellia galactanivorans*, *Zobellia uliginosa*, *Muricauda ruestringensis* and strain ACAM 210, with sequence similarities of 90.0, 90.0, 90.1, 90.1 and 89.8 %, respectively. The values of the level of 16S rDNA sequence similarities of KMM 3516^T with other members of the CFB were only 78.6–90.1 %. The large phylogenetic distance of the novel marine environmental isolate from related genera of the CFB group described to date clearly demonstrates that strain KMM 3516^T represents a new genus.

The main differential phenotypic traits of strain KMM 3516^T and members of other allied marine taxa are shown in Table 2. It is distinguished from the genus *Arenibacter* by the presence of flexirubin pigments and high DNA G+C content. KMM 3516^T and members of the genus *Zobellia* have similar characteristics in common, but *Zobellia* species are agarolytic and motile by gliding. The absence of the gliding motility, flexirubin production, starch and agar hydrolysis differentiates KMM 3516^T from *Cellulophaga* species. Strain KMM 3516^T differs from *M. ruestringensis* by respiratory metabolism, catalase, gelatinase and flexirubin production, and absence of gliding motility. Gliding motility was never observed for strain KMM 3516^T under any culture conditions used. KMM 3516^T is distinguished from the *Psychroflexus* species by the presence of flexirubin pigments, ability to grow at 43 °C and absence of starch hydrolysis. Strain KMM 3516^T is clearly differentiated from the genera *Salegentibacter*, *Polaribacter*, *Tenacibaculum*, *Gelidibacter* and *Psychroserpens* by several phenotypic traits including the presence of flexirubin pigments, oxidase activity, gliding motility, growth temperature, nitrate reduction and higher DNA G+C content (Table 2).

The fatty acid profiles of KMM 3516^T and related marine bacteria of the family *Flavobacteriaceae* (Table 1) were examined. The strains tested were grown under the same conditions (nutrient medium composition, period and temperature of cultivation) because of the results of cellular fatty acid analysis often depend on the growth conditions (Kaneda, 1991; Barbeyron *et al.*, 2001; Männistö & Puhakka, 2001). The whole-cell fatty acid data show that KMM 3516^T has similar cellular fatty acid composition with members of the family *Flavobacteriaceae*, but its fatty acid profile differs significantly from other phylogenetic relatives. As shown in Table 1, KMM 3516^T contains i15:0 and α15:0 as its major fatty acids, but has much more i15:0 (68.8 %) in comparison with the genera *Arenibacter*, *Tenacibaculum* and *Flavobacterium* (16.0, 19.6 and 7.3 %, respectively). Strain KMM 3516^T can be differentiated from members of the genera *Arenibacter*, *Zobellia*, *Salegentibacter*, *Cellulophaga* and *Flavobacterium* by the amount of 16:1ω7 (0.1 and 12.4, 10.1, 6.0, 4.9, 14.3 %, respectively). The level of i15:0-2-OH of the genus *Salegentibacter* is 29.3 % in contrast to the other above-mentioned genera and KMM 3516^T which contained <2.1 %. Low value of i17:0-3-OH fatty acid differs holothurian isolate from representatives

Fig. 1. 16S rDNA neighbour-joining phylogenetic tree showing the relationship of *Vitellibacter vladivostokensis* KMM 3516^T to other species of the CFB phylum. *Rhodothermus marinus* (GenBank no. X80994) was used as the outgroup. Percentage bootstrap value of 1000 replicates (only 70% or higher were cited) are shown the nodes. The scale bar indicates a genetic distance of 0.01 (K_{nuc}).

Table 2. Differential characteristics of *Vitellibacter vladivostokensis* KMM 3516^T and allied genera of the family Flavobacteriaceae

Taxa: 1, *Vitellibacter vladivostokensis*; 2, *Arenibacter*; 3, *Zobellia*; 4, *Muricauda*; 5, *Cellulophaga*; 6, [*Cytophaga*] *latercula*; 7, [*Cytophaga*] *marinoflava*; 8, *Flavobacterium* spp. (*F. gillisiae*, *F. tegeticola*, *F. xanthum*, *F. frigidarium*); 9, *Psychroflexus*; 10, *Salegentibacter*; 11, *Polaribacter*; 12, *Tenacibaculum*; 13, *Gelidibacter*; 14, *Psychroserpens*. Data from Ivanova *et al.* (2001), Barbeyron *et al.* (2001), Bruns *et al.* (2001), Bowman *et al.* (1997, 1998), Reichenbach (1989), McCammon & Bowman (2000), Dobson *et al.* (1993), Gosink *et al.* (1998), Bernardet & Grimont (1989), Suzuki *et al.* (2001), Humphry *et al.* (2001), and this study. Results: –, negative; +, positive; v, variable; ND, not determined; NG, no growth.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Metabolism*	A	A	A	F	A	A	A	A	A	A	A	A	A	A
Oxidase/catalase	+/+	+/+	+/+	+/-	+/+	+/+	+/+	v/+	+/+	+/+	v/+	+/+	-/+	-/+
Flexirubin	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gliding motility	+	+	+	+	+	+	+	v	v	+	+	+	+	+
Requires Na ⁺ for growth	+	+	+	+	+	+	+	+	v	+	+	+	+	+
Requires amino acids	+	+	+	+	+	+	+	+	v	+	+	+	+	+
Growth at 43 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth with 15% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from carbohydrates	+	+	+	+	+	+	+	v	+	+	+	v	+	+
Hydrolysis of:														
Agar	+	+	+	+	+	+	+	+	+	+	+	v	+	+
Casein	+	+	+	+	v	+	+	v	+	+	+	+	v	+
Gelatin	+	+	+	+	+	+	+	v	+	+	v	v	v	v
Starch	+	+	+	+	+	+	+	v	+	+	+	v	+	+
Chitin	+	+	+	+	+	+	+	v	+	+	+	v	+	+
DNase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	v	+	+	+	v	v	+	+	+	+
H ₂ S production	+	+	+	+	+	+	+	v	+	+	+	v	+	+
Nitrate reduction	+	+	+	+	v	+	+	v	+	+	v	v	+	+
G + C content (mol%)	41.3	37–38	42–43	41.4	33–38	34.0	37.0	32–36	32–36	37–38	31–33	31–33	36–38	27–29

*A, Anaerobic; F, facultatively anaerobic.

of the genera *Arenibacter* and *Cellulophaga*. Of particular interest is the fact that cells of KMM 3516^T contain 93·4 % branched fatty acids in contrast to its close phylogenetic neighbours *A. latericius* KMM 426^T and *Z. uliginosa* CIP 104808^T (61·8 % and 60·7 %, respectively).

The results of the polyphasic taxonomic analysis indicate that strain KMM 3516^T does not belong to any of the other taxa currently included in the CFB phylum. Thus, we propose that strain KMM 3516^T be classified as *Vitellibacter vladivostokensis* gen. nov., sp. nov.

Description of *Vitellibacter* gen. nov.

Vitellibacter (Vi.tel.li.bac'ter. L. n. *vitellus* egg yolk; N.L. masc. n. *bacter* rod; N.L. masc. n. *Vitellibacter* egg-yolk-coloured rod).

Rod-shaped cells, not flagellated, no gliding motility. Gram-negative. Do not form endospores. Requires Na⁺ ions for growth. Strictly aerobic. Produces non-diffusible yellow-orange pigments. Flexirubins are synthesized. Chemo-organotrophic. Cytochrome oxidase-, catalase- and alkaline phosphatase-positive. The major respiratory quinone is MK-6. The main cellular fatty acids are branched-chain saturated fatty acids i15:0 and α 15:0. As determined by 16S rDNA sequence analysis, the genus *Vitellibacter* is a member of the *Cytophaga*–*Flavobacterium*–*Bacteroides* phylum, family *Flavobacteriaceae*. The type species is *Vitellibacter vladivostokensis*.

Description of *Vitellibacter vladivostokensis* sp. nov.

Vitellibacter vladivostokensis (vla.di.vo.sto.ken'sis. N.L. adj. *vladivostokensis* pertaining to Vladivostok, a city in Asian Russia, where the organism was first isolated).

Main characteristics are otherwise the same as those given for genus. In addition, cells are 0·3–0·5 μ m wide and 3–10 μ m long. Colonies are 2–4 mm in diameter, circular, shiny, convex, with entire edges on solid media containing high nutrient components. Growth occurs at 4–43 °C. Optimal temperature for growth is 28 °C. Growth is detected at 1–6 % NaCl. Decomposes gelatin, casein, DNA, elastin, Tween 20 and Tween 40. Do not hydrolyse cellulose (CM-cellulose and filter paper), chitin, agar, starch, alginate, urea, Tween 60 and Tween 80. Does not form acid from arabinose, cellobiose, fucose, galactose, glucose, lactose, maltose, melibiose, raffinose, rhamnose, sorbose, sucrose, xylose, adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, *N*-acetylglucosamine or citrate. Glycerol, *N*-acetylglucosamine, acetate, citrate, malonate, tartrate and alanine are utilized. Arabinose, glucose, lactose, mannose, sucrose, inositol, mannitol and sorbitol are not utilized. Nitrate is not reduced to nitrite. H₂S and indole production are negative. The G + C content of the DNA is 41·3 mol%.

The type strain is deposited in Collection of Marine Microorganisms (KMM) of Pacific Institute of Bioorganic

Chemistry of Far-Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia, as KMM 3516^T, and in NITE Biological Resource Center (formerly Institute for Fermentation Osaka; IFO), Chiba, Japan, as NBRC 16718^T; isolated from the holothurian *Apostichopus japonicus*, collected in Troitsa Bay in the Gulf of Peter the Great (Sea of Japan).

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