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Ochrovirga pacifica gen. nov., sp. nov., A Novel Agar-Lytic Marine Bacterium of the Family *Flavobacteriaceae* Isolated From A Seaweed

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Abstract A strain designated as S85^T was isolated from a seaweed collected from coastal area of Chuuk State in Micronesia. The strain was gram-negative, rod-shaped, and non-motile and formed yellow colonies on the SWY agar (0.2 % yeast extract and 1.5 % agar in seawater) and Marine agar 2216. The strain grew at pH 5–9 (optimum, pH 8), at 15–40 °C (optimum, 25–28 °C), and with 1–9 % (w/v) NaCl (optimum, 3 %). The phylogenetic analysis based on 16S rRNA gene sequence showed that strain S85^T was related to *Lutibacter litoralis* CL-TF09^T and *Maritimimonas rapanae* A31^T with 91.4 % and with 90.5 % similarity, respectively. The dominant fatty acids were iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH and iso-$C_{17:0}$ 3-OH, $C_{16:0}$ 3-OH and summed feature 3 ($C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH). The major isoprenoid quinone was MK-6. The DNA G+C content of the type strain was 34.6 mol %. The major polar lipids were phosphatidylethanolamine, an unknown glycolipid and two unknown polar lipids. Based on this polyphasic taxonomic data, strain S85^T stands for a novel species of a new genus, and we propose the name *Ochrovirga pacifica* gen. nov., sp. nov. The type strain of *O. pacifica* is S85^T (=KCCM 90106 =JCM 18327^T).

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Introduction

The family *Flavobacteriaceae* [5, 27] is one of the major phylogenetic groups within the phylum *Bacteroidetes* [13]. The family is currently comprised of more than 110 genera with validly published names which were isolated from diverse marine habitats [1, 7, 8]. Many members of the family are known to be involved in degradation of various biopolymers such as cellulose, chitin, and pectin [17]. Also, some are known agar degrader [2, 3, 21–23]. Agar is the most abundant polysaccharide found in the cell walls of many red algae and is composed of agarose and agaropectin. Agarase is the hydrolytic enzyme responsible for the breakdown of agar, resulting in oligosaccharides with various bioactivities, such as antioxidant or whitening effects [25, 33]. Thus, these compounds can be useful for food, cosmetic, and medical industries [18, 35]. Previously, we analyzed the genome sequence of strain S85^T and predicted ten agar-degrading enzymes [24].

Strain S85^T, a deeply branching novel member of the family *Flavobacteriaceae*, was isolated from seaweed collected from Micronesia. In this study, polyphasic taxonomy of the strain S85^T was conducted.

Materials and Methods

Isolation and Cultivation of Bacterial Strain

An agar-degrading marine bacterium designated as strain S85^T was isolated from seaweed sample collected from coastal area (7°22′19″N, 151°35′50″E) of Chuuk State in Micronesia. The seaweed sample was diluted with autoclaved seawater, spread on SWY agar plate (0.2 % yeast extract and 1.5 % agar in seawater), and subsequently incubated at 30 °C for 1 day. The positive colonies showing pits were selected from the SWY agar plate and re-streaked on marine agar 2216 (MA, Difco). These colonies were pure cultured in marine broth 2216 (MB, Difco) at 30 °C for 1–2 days and were preserved in 20 % glycerol at -80 °C.

16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA gene of strain S85^T was amplified from a pure culture colony using bacterial primer set (primers 27F and 1492R) specific to the 16S rRNA gene and was sequenced (Macrogen Inc., Korea). The 1401-bp sequence was analyzed with BLASTN algorithm (http://blast.ncbi.nlm.nih.gov) and aligned to 16S rRNA sequences of closely related species. Alignments were carried out using the CLUSTAL W program [31], and the analysis was performed using BioEdit [15]. The distance matrix was produced on the basis of Kimura's two-parameter model [16]. Phylogenetic trees were generated by the neighbor-joining [28], maximum-parsimony [12], and maximum-likelihood [10] methods in MEGA (version 5) [30]. The topologies of the resultant trees were evaluated using bootstrap analysis of 1,000 replicates [11].

Morphological, Physiological, Biochemical, And Chemotaxonomic Characterization

The cell size and morphology of strain S85^T were examined under a scanning electron microscope (SEM) (Hitachi S-2460N, Japan). Gram reaction was determined using the Gram stain kit (bioMérieux) according to the manufacturer's instructions, and the cells were observed under a light microscope (Nikon Eclipse E200, Japan). Gliding motility was investigated as described by Bowman [6]. Catalase and oxidase tests were performed as described by Barrow & Feltham [4]. Nitrate reduction, indole production, arginine dihydrolase, urease, β-galactosidase, aesculin hydrolysis, gelatin hydrolysis, and glucose acidification were tested using the API 20NE kit (bioMérieux). Also, assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, gluconate, caprate, adipate, malate, citrate, and phenyl-acetate was tested using the same kit. Other enzymatic activities were tested using the API ZYM kit (bioMérieux). The temperature range for bacterial growth was determined by observing growth in MB incubated between 5 and 50 °C. The optimum pH for growth (range between 3 and 11, using increments of 1) was determined in 0.2 % yeast extract solution with various pH buffers (citrate/phosphate, phosphate, and glycine/NaOH buffers). Different concentrations of NaCl (0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 %) for growth were tested in 0.2 % yeast extract solution.

Isoprenoid quinone, cellular fatty acids, and the G+C content of the genomic DNA were analyzed at the Korean Culture Center of Microorganisms (KCCM, Korea). Isoprenoid quinones were extracted with chloroform/methanol (2:1) from freeze-dried cells grown in MB for 3 days at 30 °C and purified on TLC. The isoprenoid quinones were analyzed by HPLC using a symmetry reversed-phase C18 column [19]. Cellular fatty acids from cells grown on MB for 3 days at 30 °C were analyzed according to the standard protocol of the Sherlock Microbial Identification System (MIDI, version 4.0) and were subsequently compared with other type species. DNA G+C content was determined by HPLC analysis of deoxyribonucleosides as described previously [20]. Polar lipid composition analysis was conducted according to the procedure described earlier [34].

Flavobacteriaceae type species (*Lutibacter litoralis* CL-TF09^T, *Maritimimonas rapanae* A31^T, *Polaribacter filamentous* 215^T, and *Tenacibaculum maritimum* IFO 15946^T) that share more than 90 % similarities were selected for comparative analysis against the strain S85^T (Tables 1, 2). Furthermore, we tested susceptibility to antibiotics using commercially available antibiotic disks (Oxoid): amikacin (30 μg), ampicillin (10 μg), ampicillin-sulbactam (10/10 μg), amoxicillin-clavulanic acid (20/10 μg), cefoxitin (30 μg), cefuroximesodium (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), meropenem (10 μg), ofloxacin (5 μg), piperacillin (100 μg), tetracycline (30 μg), and trimethoprim-sulfamethoxazole (1.25/23.75 μg). Bacterial suspension grown for 3 days at 30 °C in shaking incubator was spread on MA plates. With antibiotic disks in place, plates were incubated for 3 days at 30 °C. Any sign of growth inhibition was recorded as indicative of antibiotic sensitivity.

Deposition of Strain and Registration of 16S rRNA Gene Sequence

Strain S85^T was deposited in the KCCM as KCCM 90106^T and in the Japan Collection of Microorganisms (JCM, Japan) as JCM 18327^T. The 16S rRNA gene sequence of strain S85^T was deposited in GenBank (http://www.ncibi.nlm.nih.gov) with accession number JN596241.

Table 1 Differential characteristics of strain S85^T and related organisms

	1	2	3	4	5
Habitat	Seaweed	Tidal flat	Veined rapa whelk	Surface seawater	Marine fish
Cell morphology	Rod	Rod	Rod	Filamentous	Rod
Gliding motility	–	–	–	–	+
Cell size (μm)	0.5 × 0.7 – 1.8	0.3 – 0.8 × 1.0 – 5.7	0.3 – 0.4 × 0.8 – 1.2	0.5 – 1.2 × 1.6 – 32	0.5 × 2 – 30
Colony color	Yellow	Yellow	Yellow	salmon	Yellow
Range for growth					
pH	5–9 (8)	7–8	5–9 (7)	ND	5.9–8.6
Temperature (°C)	8–42 (25–28)	5–30	10–37 (30)	4–19	15–34 (30)
NaCl requirement (%)	1–9 (3)	+[a]	+[b]	2.5	+[c]
Seawater requirement	–	+	+	–	+
Acid production from glucose	–	–	+	+	ND
Oxidase	–	–	+	–	+
β-galactosidase	–	ND	–	–	ND
Nitrate reduction	–	–	–	–	+
Hydrolysis of:					
Aesculin	+	+	–	W+	–
Gelatine	–	+	+	W+	+
Agar	+	ND	–	ND	ND
Growth on:					
D-Glucose	–	–	+	+	ND
D-Mannose	+	–	ND	+	–
D-Mannitol	+	–	ND	–	–
D-Maltose	+	ND	ND	–	–
Citrate	+	+	–	–	ND
N-acetyl-glucosamine	+	–	ND	–	+
G+C content (mol %)	34.6	33.9	31.7	31–33	31–32

Strain: 1, strain S85^T (Data are from this study); 2, *Lutibacter litoralis* CL-TF09^T [9]; 3, *Maritimimonas rapanae* A31^T [26]; 4, *Polaribacter filamentous* 215^T [14]; 5, *Tenacibaculum maritimum* IFO 15946^T [29, 32]. All strains are gram-negative and catalase-positive

[a] Growth occured in ZoBell's medium containing 1–5 % sea salts. However, the strain failed to grow on medium with 3 % NaCl as sole salt source

[b] Growth occured in media (ZoBell's, TSA, R2A, and NA) containing 3–6 % sea salts (optimum 4–5 %), but not in media with NaCl (1–12 %) as the sole salt source

[c] Growth occurs in media containing seawater (30–100 %), but not in media with NaCl alone as the sole salt source

+ Positive; − Negative; *ND* not detected; *W* Weak result

Results and Discussion

Phylogenetic Analysis

Phylogenetic trees were generated by neighbor-joining, maximum-parsimony, and maximum-likelihood methods (Fig. 1). A total of 22 strains were compared with S85^T, which all belongs to the family *Flavobacteriaceae*. The 16S rRNA gene sequence of *Chryseobacterium antarcticum* AT1013^T was used as an outgroup. Strain S85^T produces a phyletic line with genus *Lutibacter* (91.4–90.3 %), *M. rapanae* (90.5 %), genera *Polaribacter* (90.5–89.3 %), *Tenacibaculum* (90.6–89.1 %), and so on. The phylogenetic result implied that the strain is a novel genus and novel species belonging to the family *Flavobacteriaceae*.

Phenotypic and Chemotaxonomic Characteristics

According to the previous reports of Reichenbach [27] and Bernardet [5], most of the known species in the family *Flavobacteriaceae* were Gram-negative and rod-shaped, possessed menaquinone 6 (MK-6) as the only respiratory quinone or the major respiratory quinone, and its G+C contents were composed of 27–44 mol %. The bacterial isolate in this study also represented similar phenotypic and chemotaxonomic characteristics. Strain S85^T formed yellow and undulate colonies on the SWY agar and MA plate. The bacterium was Gram-negative and non-motile, and SEM observation revealed that the non-flagellated cells were rod-shaped with the size range of 0.7–1.8 μm, predominantly 1.3 μm (length) × 0.5 μm (width) (Fig. 2).

Table 2 Cellular fatty acid compositions (%) of strain S85^T and related organisms of the family *Flavobacteriaceae*

Fatty acid	1	2	3	4	5
Saturated					
$C_{15:0}$	1.6	1.7	3.3		
$C_{16:0}$	4.5		3.8		Tr
$C_{18:0}$		1.3	3.1		1.2
Saturated branched-chain					
iso-$C_{13:0}$	4.4		Tr	5	1.5
iso-$C_{14:0}$		2.2	Tr		1.0
iso-$C_{15:0}$	23.4	16.7	20.1	22	18.8
iso-$C_{16:0}$			2.5		Tr
iso-$C_{16:1}$					1.7
anteiso-$C_{13:0}$	tr	1.4			
anteiso-$C_{15:0}$	tr	15.1	1.3	6	1.3
Unsaturated					
$C_{15:1}$ ω6c	tr	1.5		9	
$C_{17:1}$ ω6c		1.0	tr		1.0
Unsaturated branched-chain					
iso-$C_{15:1}$ G	4.4	4.2	24.2	12	11.8
iso-$C_{15:1}$ I				6	
iso-$C_{15:1}$ H				6	
iso-$C_{16:1}$ H		1.3			
anteiso-$C_{15:1}$-A		1.6	tr		
Hydroxy					
$C_{13:0}$ 3-OH				6	
$C_{15:0}$ 2-OH		2.1	Tr		Tr
$C_{15:0}$ 3-OH	tr	1.5	Tr	2	2.2
$C_{16:0}$ 3-OH	7.2	2.8	2.2		1.2
$C_{17:0}$ 2-OH	tr	3.6	Tr		
iso-$C_{15:0}$ 3-OH	14.9	17.4	7.8	22	12.9
iso-$C_{16:0}$ 3-OH		13.4	4.5		5.1
iso-$C_{17:0}$ 3-OH	9.4	3.9	11.2		7.5
Summed features					
Summed feature 3	17.4	1.0	7.5		17.6

Strain: 1, strain S85^T (Data are from this study); 2, *Lutibacter litoralis* CL-TF09^T [9]; 3, *Maritimimonas rapanae* A31^T [26]; 4, *Polaribacter filamentous* 215^T [14]; 5, *Tenacibaculum maritimum* IFO 15946^T [29, 32]. Tr was trace amount of <1 %. Summed feature 3 was $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH

Cell growth was observed at 8–42 °C (with optimum range: 25–28 °C), at pH 5.0–9.0 (with optimum pH 8), and in the presence of 0.5–9 % (w/v) NaCl (optimum salinity: 3 %). The G+C content of strain S85^T was 34.6 mol %, and the major isoprenoid quinone was MK-6. Significantly, strain S85^T has some difference compared with other type species on carbon utilization and growth factor. S85^T was oxidase negative, whereas *Maritimimonas rapanae* A31^T and *Tenacibaculum maritimum* IFO 15946^T were positive. Also, strain S85^T was capable of utilizing D-mannitol for growth, in contrast to *Lutibacter litoralis* CL-TF09^T, *Polaribacter filamentous* 215^T, and *Tenacibaculum maritimum* IFO 15946^T. Gelatin was not hydrolyzed by strain S85^T, whereas the compared type species hydrolyzed gelatin. In addition, aesculin and agar were hydrolyzed by strain S85^T. Morphological, Physiological, and biochemical characteristics differentiate S85^T from related genera which are described in Table 1.

Our strain did not show resistance to antibiotics except amikacin (30 μg). The predominant fatty acids (>5 %) were iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$ 3-OH, and $C_{16:0}$ 3-OH and summed feature 3 comprised of $C_{16:1}$ ω7c and/or iso-$C_{15:0}$ 2-OH. The fatty acid profile of strain S85^T was clearly distinguishable from type species of the genera *Lutibacter, Maritimimonas, Polaribacter,* and *Tenacibaculum* (Table 2). The major distinctive difference was the abundance of $C_{16:0}$ 3-OH in strain S85^T. The major polar lipids detected in strain S85^T were phosphatidylethanolamine, an unknown glycolipid and two unknown polar lipids.

In conclusion, strain S85^T is considered to represent a novel species in the novel genus on the basis of phylogenetic analysis using the 16S rRNA gene sequence and phenotypic and fatty acid profiles. Here, we propose the name *Ochrovirga pacifica* gen. nov., sp. nov. for strain S85^T.

Description of Ochrovirga gen. nov

Ochrovirga (O.chro.vir'ga. Gr. adj. *ochro* pale yellow; L. fem. n. *virga* rod; N. L. fem. n. *Ochrovirga* yellow-colored rod).

Cells are Gram-negative, non-motile rod, catalase-positive, and oxidase- negative. The DNA G+C content is 34.6 mol %. The predominant fatty acids are iso-$C_{15:0}$, iso-$C_{15:0}$ 3-OH, iso-$C_{17:0}$ 3-OH, and $C_{16:0}$ 3-OH and summed feature 3. The major polar lipids are phosphatidylethanolamine, an unknown glycolipid and two unknown polar lipids. The major isoprenoid quinone is MK-6. Phylogenetically, this genus is a member of the family *Flavobacteriaceae*. The type species is *O. pacifica*.

Description of O. pacifica sp. nov

Ochrovirga pacifica (pa.ci'fi.ca. L. fem. adj. *pacifica*, peaceful, referring to the Pacific Ocean, the origin of the type strain).

Cells are rod-shaped with 0.5 μm in width and 0.7–1.8 μm (mainly 1.3 μm) in length. Cell growth occurs in MB at 8-42 °C, at pH 5.0–9.0, and in the presence of 1–9 % (w/v) NaCl. Nitrate is not reduced to nitrite. Indole is not produced. Acid is not produced from glucose. Cytochrome oxidase, arginine dihydrolase, urease, and

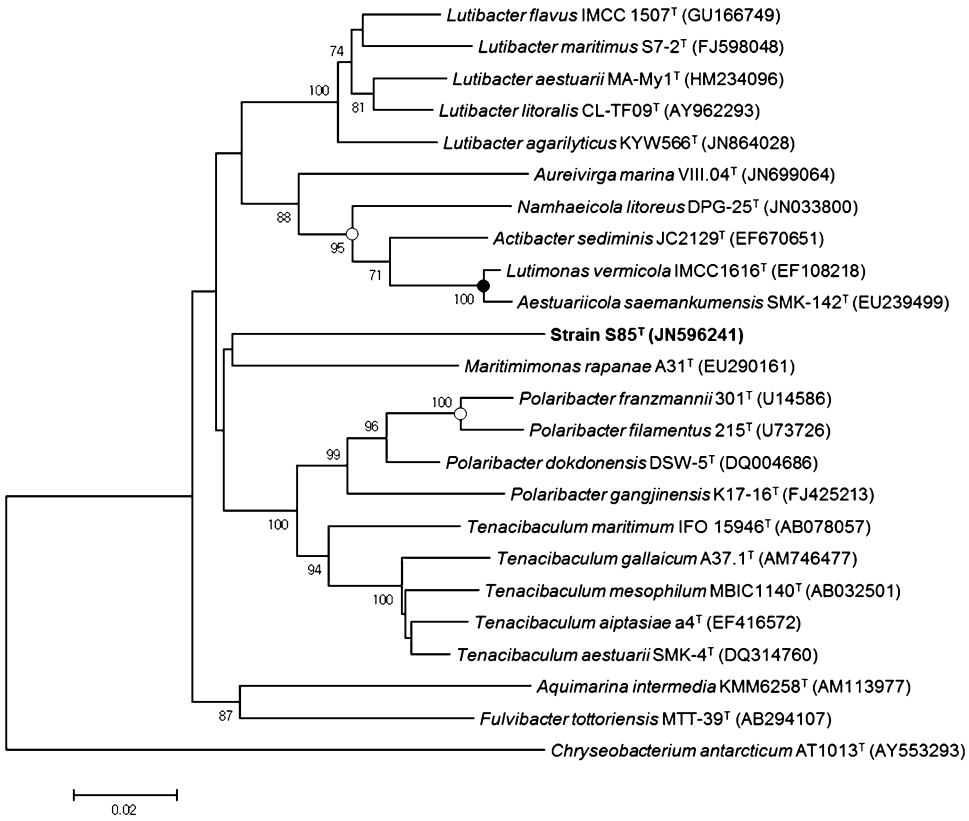

Fig. 1 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain S85^T and related taxa. Bootstrap values higher than 70 % by 1,000 replications are indicated at nodes. *Bar* represents 0.02 substitutions per nucleotide position. The sequence of *Chryseobacterium antarcticum* AT1013^T was used as an outgroup. *Black circle* recovered by the maximum-likelihood and maximum-parsimony trees. *White circles* recovered by the maximum-likelihood tree

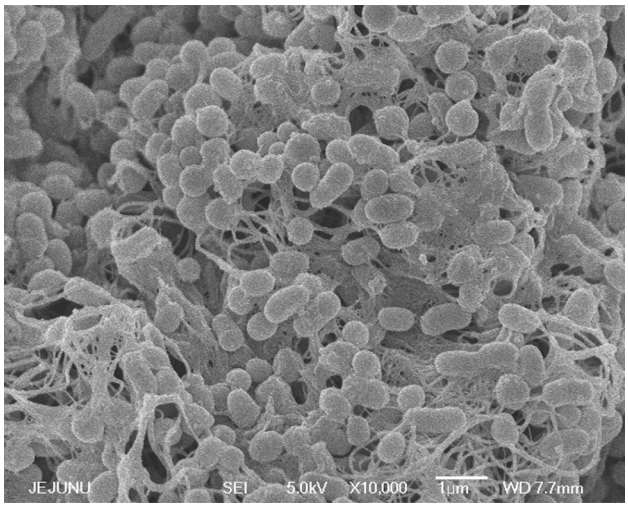

Fig. 2 Scanning electron micrograph of strain S85^T. Cells were grown at 30 °C on marine agar for 1 day. *Scale bar* was 1 μm

β-galactosidase activities are absent. Aesculin and agar are degraded, but gelatin is not. D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, adipate, malate, and citrate are assimilated, but D-glucose, L-arabinose, caprate, and phenyl-acetate are not. Alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, and α-glucosidase activities are present. Esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities are absent.

The type strain, S85^T (=KCCM 90106^T =JCM 18327^T), was isolated from a seaweed collected from coastal area (7°22′19″N, 151°35′50″E) of Chuuk State in Micronesia.

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