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Dokdonia flava sp. nov., isolated from the seaweed *Zostera marina*

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Abstract

A non-motile, proteorhodopsin-containing, yellow and rod-shaped bacterial strain, designated ZODW10^T, was isolated from the seaweed *Zostera marina* collected from the West Sea, Republic of Korea. Cells were Gram-stain-negative, aerobic and non-motile. The isolate required sea salts for growth. A carotenoid pigment was produced. A phylogenetic tree based on 16S rRNA gene sequences showed that strain ZODW10^T forms an evolutionary lineage within the radiation enclosing members of the genus *Dokdonia* with *Dokdonia diaphoros* CIP 108745^T (96.7 % sequence similarity) as its nearest neighbour. The major fatty acids were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{15:1}$ G. Strain ZODW10^T contained menaquinone 6 (MK-6) and phosphatidylethanolamine, an unidentified aminolipid and an unidentified polar lipid as the only isoprenoid quinone and the major polar lipids, respectively. The DNA G+C content of strain ZODW10^T was 36 mol%. On the basis of the present polyphasic characterization, it is suggested that the isolate represents a novel species of the genus *Dokdonia*, for which the name *Dokdonia flava* sp. nov. (type strain, ZODW10^T=KCTC 52953^T=JCM 32293^T) is proposed.

The genus *Dokdonia*, a member of the family *Flavobacteriaceae* in the phylum *Bacteroidetes*, was proposed by Yoon *et al.* [1] with the description of the type species, *Dokdonia donghaensis*, isolated from seawater. Subsequently, three *Krokinobacter* species, *K. genikus*, *K. diaphorus* and *K. eikastus*, isolated from marine sediment [2] were reclassified as *Dokdonia genika*, *Dokdonia diaphoros* and *Dokdonia eikasta*, respectively [3], and the description of the genus *Dokdonia* was also emended. At the time of writing, the genus *Dokdonia* comprises six species with valid names. Members of the genus are Gram-stain-negative, aerobic, non-spore-forming, non-motile and rod-shaped and contain menaquinone 6 (MK-6) as the predominant menaquinone and iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{15:1}$ G as major fatty acids [1, 3]. In the course of estimation of the bacterial diversity in a bed of seaweed, a yellow-pigmented and rod-shaped bacterial strain, designated ZODW10^T, was isolated and subjected to a taxonomic investigation.

Strain ZODW10^T was isolated from the seaweed *Zostera marina*, collected from a natural seaweed bed in the West Sea (36° 55′ 48.0″ N 126° 21′ 36.0″ E), Republic of Korea. The natural seaweed bed consisted of diverse species including *Zostera marina* and *Sargassum fulvellum*. A seaweed sample was wiped with sterilized tissue and immersed in 0.85 % (w/v) saline. The immersion sample was rotated for 30 min at 4 °C. The saline supernatant was inoculated onto marine agar 2216 (MA; Becton Dickinson) and incubated for 5 days at 25 °C. The isolate was routinely cultured on MA and preserved at −80 °C as a suspension in marine broth 2216 (MB; Becton Dickinson) containing 20 % (v/v) glycerol. Reference strains *Dokdonia pacifica* KCTC 52761^T (purchased from the Korean Collection for Type Cultures; KCTC), *D. diaphoros* CIP 108745^T, *D. eikasta* CIP 108743^T, *D. genika* CIP 108744^T (purchased from the Collection de l'Institut Pasteur; CIP), *D. donghaensis* DSW-1^T (original isolate; [1]) and *D. lutea* SFD34^T (original isolate; [4]) were used to compare the physiological and chemical properties.

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously [5]. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon-e server [6] and BLAST search program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The novel sequence and related sequences were aligned by using CLUSTAL W [7], and the alignment was refined using BioEdit version 7.2.0 [8]. Phylogenetic analysis was performed by using MEGA version 7.0 [9]. Phylogenetic trees were inferred using the

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Three supplementary figures are available with the online version of this article.

neighbour-joining [10], maximum-likelihood [11] and maximum-parsimony [12] algorithms. The distance matrix of the neighbour-joining method was generated according to the model of Jukes and Cantor [13]. The robustness of the topology in the neighbour-joining phylogenetic tree was evaluated by bootstrap analyses [14] based on 1000 resamplings.

The 16S rRNA gene sequence of strain ZODW10^T was a continuous stretch of 1447 nt. Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that the isolate was closely related to members of the genus *Dokdonia*. The closest relatives of strain ZODW10^T were *D. diaphoros* CIP 108745^T, *D. eikasta* CIP 108743^T (96.7 % sequence similarity each) and *D. donghaensis* DSW-1^T (96.4 %). 16S rRNA gene sequence similarity between strain ZODW10^T and other members of the genus *Dokdonia* was less than 96.0 %. This relationship between the new isolate and other members of the genus *Dokdonia* was also evident in all the phylogenetic trees reconstructed using the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms (Fig. 1). It is clear from 16S rRNA gene sequence similarity data and phylogenetic analysis that strain ZODW10^T represents a distinct novel species in the genus *Dokdonia*.

Growth on various standard bacteriological media was tested by using nutrient agar (NA; Becton Dickinson), Reasoner's 2A (R2A; Becton Dickinson) agar, plate-count agar (PCA; Becton Dickinson), tryptic soy agar (TSA; Becton Dickinson) and Zobell's agar [15]. Cells grown on MA at 25 °C for 2–3 days were used for physiological and biochemical tests. The Gram reaction test was performed by using the bioMérieux Gram stain kit according to the manufacturer's instructions and the Ryu non-staining KOH method [16]. Cell morphology was observed by phase-contrast

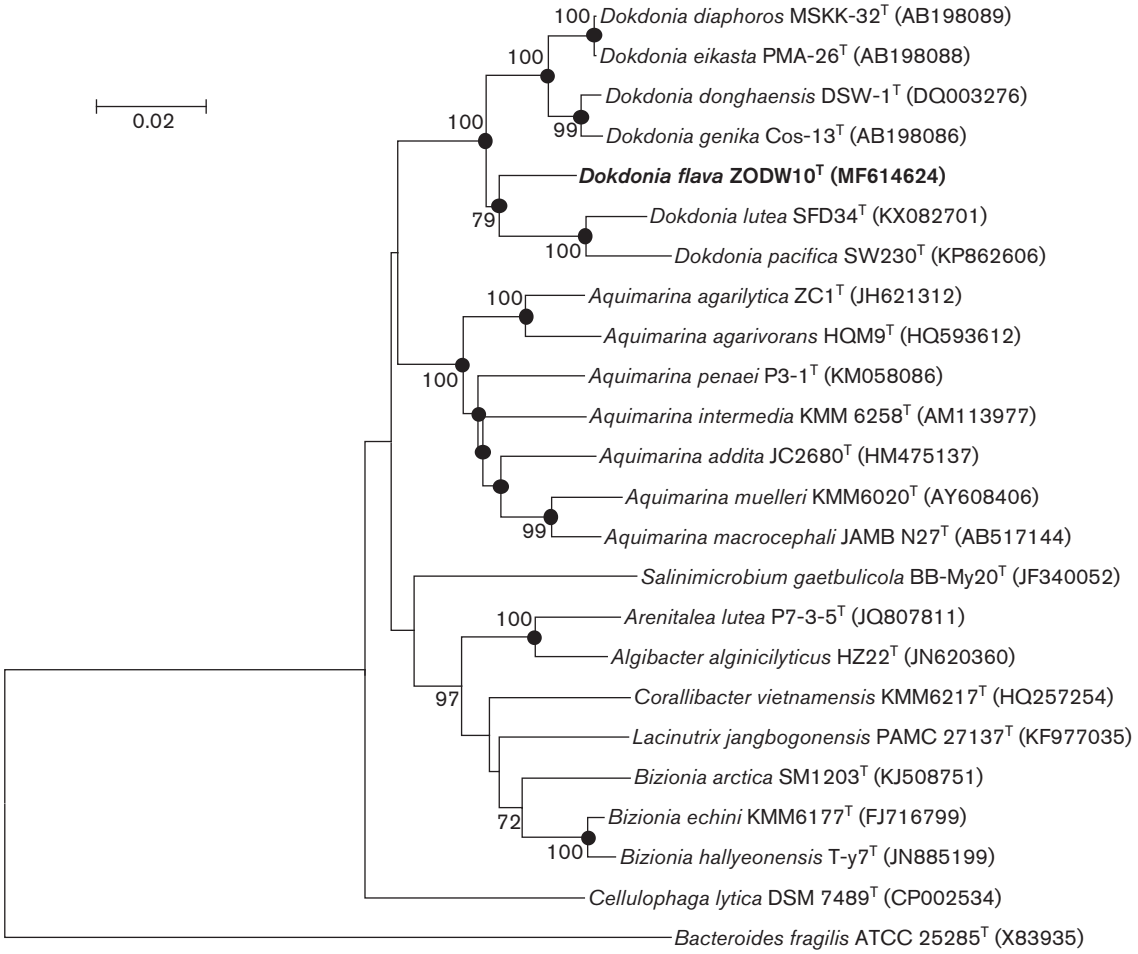

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the position of strain ZODW10^T within the family *Flavobacteriaceae*. Numbers at nodes are the levels of bootstrap support (>70 %) based on neighbour-joining analyses of 1000 resampled data sets. The sequence of *Bacteroides fragilis* ATCC 25285^T (X83935) was used as an outgroup. Closed circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. Bar, 0.02 nucleotide substitutions per position.

(ICC50; Leika) and transmission electron microscopy (CM-20; Philips) using cells grown at 25 °C for 3 days on MA. Motility was examined by observing cells grown in wet mounts using a phase-contrast microscope (ICC50; Leika). Flagellation was determined with a transmission electron microscope (CM-20; Philips) using cells cultured for 3 days in MB.

The requirement for and tolerance of sea salts (Sigma) or NaCl [final concentration: 0–10 % (w/v), using increments of 1 %] for growth was tested on ZoBell's agar and on MA prepared without NaCl. The growth experiment at pH 4–11 (increments of 1 pH units) was performed using MB containing 100 mM acetate buffer, 100 mM NaH_2PO_4/Na_2HPO_4 buffer and 100 mM $NaHCO_3/Na_2CO_3$ buffer, at pH 4–5, 6–8 and 9–11, respectively. The optimal temperature and temperature range for growth were tested on MA at 4, 7, 10, 15, 20, 25, 30, 32, 35, 40 and 45 °C. Anaerobic growth was tested on MA in a jar containing AnaeroPack-Anaero (Mitsubishi Gas Chemical), which works as an oxygen absorber and CO_2 generator, for up to 10 days.

Catalase and oxidase activities were tested in 3 % (v/v) hydrogen peroxide and oxidase reagent (bioMérieux), respectively. Acid production from sugars was tested as described by Yamaguchi and Yokoe [17]. Simmon's citrate test was carried out in Simmons' citrate agar (Sigma). H_2S production was determined on Kligler iron agar (Becton Dickinson) according to Smibert and Krieg [18]. Degradation of the following macromolecules was tested using MA as the basal medium and incubation at 25 °C for 10 days: alginate (0.5 %, w/v), CM-cellulose (1 %, w/v), casein (2 %, w/v, skimmed milk), chitin (1 %, w/v, colloidal chitin), starch (0.5 %, w/v), Tween 20 (1 %, w/v), Tween 80 (1 %, w/v), L-tyrosine (0.5 %, w/v) and xylan (1 %, w/v). Degradation was revealed by formation of clear zones around the colonies either directly [19] or after flooding with adequate staining solutions [18]. Decomposition of xylan (1 %, w/v) was tested using MA as the basal medium [20]. DNase activity was determined with DNase test agar (Becton Dickinson). The presence of flexirubin-type pigments was tested using the KOH test as described by Bernardet *et al.* [21]. Other biochemical tests and enzyme activities were performed using the API 20NE and API ZYM kits (bioMérieux) prepared according to the manufacturer's instructions except that bacterial strains were suspended in distilled water supplemented with 4 % sea salts. The proteorhodopsin gene was detected by PCR amplification as described by Yoshizawa *et al.* [22] using primers PR-Flavo-F and PR-Flavo-R. Antibiotic resistance was determined with the disc diffusion method [23] using commercial antibiotic-impregnated discs (Becton Dickinson). After 5 days of incubation at 25 °C on MA, the results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [24].

Cell extract for carotenoid pigment analysis was prepared with methanol [25] and the absorption spectrum (200–800 nm) was recorded using a UV/VIS spectrophotometer (Ultrospec 2100 pro; Biochrom). A methanol extract showed the spectrum typical of carotenoid pigments with maximum absorption at 455 and 478 nm, which closely resembled that of authentic β-carotene reported in the literature, suggesting that strain ZODW10^T could contain β-carotene or a carotenoid structurally similar to β-carotene as a dominant pigment [26].

The isolate was rod-shaped (Fig. S1, available in the online version of this article) with yellow-pigmented colonies. The isolate could not grow on sea-salts-free ZoBell's medium supplemented with NaCl and required sea salts for growth. Strain ZODW10^T was sensitive to the following antibiotics (µg per disc, unless otherwise indicated): amikacin (30), ampicillin (10), gentamicin (10), kanamycin (30), polymyxin B (300 IU) and streptomycin (10), but resistant to chloramphenicol (30), erythromycin (15), nalidixic acid (30), penicillin (10 IU), tetracycline (30) and vancomycin (30). The proteorhodopsin gene was detected from strain ZODW10^T, as in *D. donghaensis* DSW-1^T [27], *D. eikasta* CIP 108743^T and *D. genika* CIP 108744^T (Fig. S2). The detailed results of physiological and biochemical analyses are given in Table 1 and the species description. It is evident from Table 1 that there are several phenotypic characters that readily differentiate between strain ZODW10^T and phylogenetically related species. In particular, strain ZODW10^T could not grow at or with more than 6 % (w/v) sea salts.

For cellular fatty acid analysis, strain ZODW10^T and the reference strains were grown on MA and harvested at the late exponential growth phase, i.e. after 2 days at 25 °C. Extraction of fatty acid methyl esters and separation by GC were performed by using the Instant FAME method of the Microbial Identification System (MIDI) version 6.1 and the TSBA6 database. For analyses of polar lipids and isoprenoid quinones, cells grown in MB for 3 days at 25 °C were harvested and freeze-dried. Polar lipids were extracted and separated by two-dimensional TLC according to the procedures described by Minnikin *et al.* [28]. Individual lipids were identified by spraying the plates with appropriate detection reagents such as ethanolic molybdophosphoric acid, molybdenum blue, ninhydrin and α-naphthol to detect total polar lipids, phospholipids, aminolipids and glycolipids, respectively [29]. Isoprenoid quinones were extracted and purified according to Minnikin *et al.* [28] and analysed by TLC as described by Collins [30]. For DNA G+C content calculations, the DNA sample was prepared in triplicate and values were determined by the thermal denaturation method of Marmur and Doty [31].

The fatty acid profiles of strain ZODW10^T and the reference strains are described in Table 2. The major fatty acids (>10.0 % of the total) were iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH. This fatty acid profile was similar to those reported for the genus *Dokdonia*. The only isoprenoid quinone was MK-6, which is the characteristic respiratory quinone of the genus *Dokdonia* [1]. The major polar lipids of strain ZODW10^T were phosphatidylethanolamine (PE), an unidentified aminolipid (AL1) and an unidentified polar

Table 1. Phenotypic characteristics that differentiate strain ZODW10^T from other *Dokdonia* species

Strains: 1, ZODW10^T; 2, *D. diaphoros* CIP 108745^T; 3, *D. donghaensis* DSW-1^T; 4, *D. eikasta* CIP 108743^T; 5, *D. genika* CIP108744^T; 6, *D. lutea* SFD34^T; 7, *D. pacifica* KCTC 52761^T. Data are from this study unless otherwise indicated. +, Positive; –, negative. All strains showed aerobic metabolism and were positive for activity of alkaline phosphatase, catalase, esterase (C4), esterase lipase (C8), leucine arylamidase and valine arylamidase and hydrolysis of Tween 80. All strains were negative for gliding motility, production of flexirubin-type pigments, H_2S and indole, reduction of nitrate to nitrite, hydrolysis of arginine, chitin, DNA, urea and xylan, acid production from fructose, galactose, mannitol, mannose, rhamnose, trehalose and xylose, and activity of α-chymotrypsin, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase, lipase (C14) and α-mannosidase.

Characteristic	1	2	3	4	5	6	7
Growth at:							
4 °C	–	–	+	–	–	–	–
35 °C	–	–	+	–	+	–	+
pH 5	+	–	–	–	–	+	+
pH 9	–	–	–	–	–	+	+
1 % (w/v) sea salts	–	+	–	+	+	–	–
6 % (w/v) sea salts	–	+	+	+	+	+	+
Oxidase	+	+	+	+	+	–	+
Hydrolysis of:							
Alginate	–	–	–	–	–	–	+
Casein	–	+	+	+	+	–	–
CM-cellulose	–	+	–	–	–	–	+
Aesculin	+	+	+	+	+	–	+
Starch	+	–	–	–	–	+	+
Tween 20	+	+	+	+	+	–	–
L-Tyrosine	–	–	–	+	–	–	+
Gelatin	–	+	–	–	–	+	–
Enzyme activity (API ZYM):							
N-Acetyl-β-glucosaminidase	+	–	–	–	–	+	–
Acid phosphatase	+	+	–	+	+	+	+
Cystine arylamidase	–	–	–	+	+	+	–
α-Glucosidase	–	–	–	+	+	–	–
Naphthol-AS-BI-phosphohydrolase	+	+	–	+	+	+	+
Trypsin	+	+	–	–	–	+	–
Acid production from:							
Glucose	–	–	–	+	+	–	–
Lactose	–	–	–	–	+	–	–
Maltose	–	–	–	+	–	–	–
Sucrose	–	–	–	+	+	–	–
DNA G+C content (mol%)	36	33[a]	38[b]	38[a]	37[a]	35[c]	36[d]

*Data from: *a*, Khan *et al.* [2]; *b*, Yoon *et al.* [1]; *c*, Choi *et al.* [4]; *d*, Zhang *et al.* [32].

lipid (L1) (Fig. S3). The major polar lipid composition of strain ZODW10^T was similar to that of *D. pacifica* [32]. However, the major polar lipid composition of the new isolate was different from those of *D. diaphoros*, *D. donghaensis, D. eikasta* and *D. genika* [3] in the presence of an unidentified aminolipid at a different position, and from that of *D. lutea* in the absence of two aminophospholipids. The DNA G+C content of strain ZODW10^T was 36 mol%, which falls within the range reported for members of the genus *Dokdonia* (33–38 mol%) [1, 2, 4, 28].

On the basis of data from the polyphasic study presented here, it is evident that strain ZODW10^T represents a novel species in the genus *Dokdonia*, for which the name *Dokdonia flava* sp. nov. is proposed.

DESCRIPTION OF *DOKDONIA FLAVA* SP. NOV.

Dokdonia flava (fla′va. L. fem. adj. *flava* yellow, pertaining to the yellow colour of the colonies).

Cells are Gram-stain-negative, aerobic, non-motile, rod-shaped and approximately 0.80–0.89 µm in diameter and 2.24–3.84 µm in length. Colonies are circular, convex, smooth, 1–2 mm in diameter and yellow on MA after 3 days. Growth occurs on MA but not on NA, PCA, R2A agar or TSA. Requires sea salts at concentrations of 2–5 % (w/v)

Table 2. Cellular fatty acid composition (%) of strain ZODW10^T and the type strains of *Dokdonia* species

Strains: 1, ZODW10^T; 2, *D. diaphoros* CIP 108745^T; 3, *D. donghaensis* DSW-1^T; 4, *D. eikasta* CIP 108743^T; 5, *D. genika* CIP108744^T; 6, *D. lutea* SFD34^T; 7, *D. pacifica* KCTC 52761^T. Data are from this study. —, Not detected; TR, trace amount (<1 %). For fatty acid analysis, all strains studied were grown on MA at 25 °C for 2 days. Fatty acids amounting to <1 % of the total fatty acids in all strains are omitted.

Fatty acid	1	2	3	4	5	6	7
Straight-chain saturated							
$C_{15:0}$	1.1	4.2	TR	1.1	TR	TR	1.2
$C_{16:0}$	TR	2.3	TR	TR	TR	2.1	TR
$C_{18:0}$	–	–	–	–	–	1.6	–
$C_{15:0}$ 2-OH	1.9	2.4	TR	1.4	1.4	TR	–
$C_{16:0}$ 3-OH	TR	–	TR	3.0	1.9	TR	1.0
$C_{17:0}$ 2-OH	4.7	7.4	4.9	4.9	6.7	1.3	TR
Branched saturated							
iso-$C_{13:0}$	1.1	TR	TR	TR	TR	TR	TR
iso-$C_{14:0}$	1.2	2.5	1.1	TR	1.3	TR	TR
iso-$C_{15:0}$	26.9	16.9	25.5	27.5	18.8	30.4	33.7
iso-$C_{16:0}$	TR	5.5	4.8	4.1	4.8	1.1	TR
iso-$C_{15:0}$ 3-OH	8.9	3.2	2.4	4.1	3.0	6.5	9.5
iso-$C_{16:0}$ 3-OH	5.0	13.6	5.6	5.9	7.4	2.8	2.3
iso-$C_{17:0}$ 3-OH	17.9	12.6	12.9	15.9	13.5	22.2	24.8
anteiso-$C_{15:1}$ A	1.5	1.1	1.6	1.2	2.5	TR	–
anteiso-$C_{15:0}$	6.6	8.1	6.3	8.1	7.4	1.1	1.1
Monounsaturated							
iso-$C_{15:1}$ G	18.6	6.5	19.1	10.9	16.4	17.7	14.6
iso-$C_{16:1}$ G	–	1.8	TR	–	TR	–	–
$C_{20:4}\omega6,9,12,15c$	–	1.6	–	3.2	–	–	–
Summed feature 3*	–	3.6	9.0	3.0	8.7	2.3	5.1
Summed feature 4*	–	–	–	–	–	1.1	–
Unknown 13.565	1.3	TR	TR	1.0	TR	2.1	2.0
Unknown 16.582	TR	TR	1.0	1.2	TR	TR	1.5

*Summed features are groups of two or three fatty acids that cannot be separated by GC with the MIDI system. Summed feature 3 contained $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

(optimum, 4 %) for growth and growth does not occur on sea-salts-free ZoBell's medium supplemented with NaCl only. Growth occurs at pH 5–8 (optimum, pH 7) and at 10–30 °C (optimum, 25 °C). Catalase- and oxidase-positive. Flexirubin-type pigments are absent. Contains proteorhodopsin. Methanol extracts show the spectrum typical of carotenoid pigments with maximum absorption at 455 and 478 nm. Indole and H_2S are not produced. Aesculin, starch, Tween 20 and Tween 80 are hydrolysed, but alginate, arginine, casein, chitin, CM-cellulose, DNA, gelatin, L-tyrosine, urea and xylan are not. Negative for assimilation of adipic acid, arabinose, capric acid, glucose, malic acid, maltose, mannitol, mannose, phenylacetic acid, potassium gluconate, trisodium citrate and *N*-acetyl-glucosamine. Negative for acid production from fructose, galactose, glucose, mannose, lactose, maltose, mannitol, rhamnose, sucrose, xylose and trehalose. In the API ZYM gallery, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), *N*-acetyl-β-glucosaminidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are present but α-chymotrypsin, cystine aryamidase, α-fucosidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-glucuronidase, lipase (C14) and α-mannosidase activities are absent. The predominant fatty acids (>10.0 % of the total fatty acids) are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{15:1}$ G. The only respiratory quinone detected is MK-6. The major polar lipids are phosphatidylethanolamine, an unidentified aminolipid and an unidentified polar lipid; two unidentified aminolipids and an unidentified polar lipid are also present.

The type strain is ZODW10^T (=KCTC 52953^T=JCM 32293^T), isolated from *Zostera marina* collected from the West Sea, Republic of Korea. The DNA G+C content of the type strain is 36 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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