Microbulbifer marinus sp. nov. and *Microbulbifer yueqingensis* sp. nov., isolated from marine sediment

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Two Gram-negative, aerobic strains, Y215^T and Y226^T, were isolated from sediment from Yueqing Bay, Zhejiang Province, China. The two novel strains were both positive for oxidase activity, nitrate reduction, and aesculin and casein decomposition, but negative for gelatin and tyrosine decomposition. Catalase activity, and starch and Tween 80 decomposition differed between the two strains. Cells of both novel strains were rod-shaped in young cultures and ovoid in older cultures. Optimum NaCl concentration and pH range for growth of both strains were 2.0–3.0 % (w/v) and 7.0–8.0, respectively, whereas the optimum growth temperature for strain Y215^T (25–30 °C) was lower than that for strain Y226^T (30–37 °C). The genomic DNA G+C contents of strains Y215^T and Y226^T were 54.0 and 56.7 mol%, respectively. The major fatty acids in both isolates were iso-$C_{15:0}$ and iso-$C_{17:1}\omega9c$, which was also the case in the reference strains apart from *Microbulbifer salipaludis*, which possessed $C_{18:1}\omega7c$ as the predominant fatty acid. The predominant isoprenoid quinone was Q-8 and the major polar lipids of both strains were phosphatidylethanolamine, phosphatidylglycerol and an unknown glycolipid. Both strains had highest 16S rRNA gene sequence similarity to members of the genus *Microbulbifer*. Strain Y215^T was closely related to the type strains of *Microbulbifer maritimus* (97.6 %) and *Microbulbifer donghaiensis* (97.5 %), whereas strain Y226^T was closely related to the type strain of *M. salipaludis* (97.6 %). Phylogenetic analysis based on 16S rRNA gene sequences showed that strains Y215^T and Y226^T fell into two separate clusters. The DNA–DNA relatedness values of strain Y215^T with *M. maritimus* TF-17^T and *M. donghaiensis* CN85^T were 34.1 and 32.8 %, respectively, whereas that between strain Y226^T and *M. salipaludis* SM-1^T was 38.0 %; these values are significantly lower than the threshold value for the delineation of bacterial species. On the basis of their distinct taxonomic characteristics, the two isolates represent two novel species of the genus *Microbulbifer*, for which the names *Microbulbifer marinus* sp. nov. and *Microbulbifer yueqingensis* sp. nov. are proposed; the type strains are Y215^T (=CGMCC 1.10657^T=JCM 17211^T) and Y226^T (=CGMCC 1.10658^T=JCM 17212^T), respectively.

The genus *Microbulbifer* was first proposed in 1997 to accommodate a Gram-negative, strictly aerobic gamma-proteobacterium capable of utilizing a variety of hydrocarbons (González *et al.*, 1997), and most species of the genus *Microbulbifer* possess a rod–coccus cell cycle in association with the growth phase (Nishijima *et al.*, 2009). The type species, *Microbulbifer hydrolyticus*, was isolated from marine pulp mill effluent enrichment cultures from a paper factory. Subsequently, at the time of writing, nine further species, *Microbulbifer salipaludis* (Yoon *et al.*, 2003a), *M. maritimus* (Yoon *et al.*, 2004), *Microbulbifer celer* (Yoon *et al.*, 2007), *Microbulbifer halophilus* (Tang *et al.*, 2008), *Microbulbifer agarilyticus* and *Microbulbifer thermotolerans* (Miyazaki *et al.*, 2008), *Microbulbifer epialgicus* and *Microbulbifer variabilis* (Nishijima *et al.*, 2009), and *Microbulbifer donghaiensis* (Wang *et al.*, 2009) have been described and *Pseudomonas elongata* has been reclassified as *Microbulbifer elongatus* (Yoon *et al.*,

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Y215^T and Y226^T are GQ262812 and GQ262813, respectively.

A supplementary table and three supplementary figures are available with the online version of this paper.

2003b). Here, we present a polyphasic study describing two novel *Microbulbifer* strains isolated from sediment from Yueqing Bay, Zhejiang Province, China.

A sediment sample was collected from Yueqing Bay in Zhejiang Province, China (28° 11′ 41″ N 121° 07′ 00″ E), in February 2009. Approximately 100 mg subsample was suspended in 3 ml sterile seawater and vortexed for 15 min. The dispersed sediment suspension was diluted and added to marine agar 2216 (MA; Difco). After 3 days aerobic incubation at 25 °C, two colonies, designated Y215^T and Y226^T, were chosen for further characterization. The novel strains were purified by repeated restreaking and maintained on MA. Three *Microbulbifer* species were used as reference strains for DNA–DNA hybridization and fatty acid analysis: *M. salipaludis* and *M. maritimus* were obtained from the Japan Collection of Microorganisms, Saitama, Japan, and *M. donghaiensis* was obtained from the previous study (Wang *et al.*, 2009). All three species mentioned above and *M. hydrolyticus*, obtained from a previous study (González *et al.*, 1997), were used for comparison of phenotypic characteristics.

Cell morphology and motility were examined by means of light microscopy and transmission electron microscopy after 2 days incubation at 30 °C on MA. Cells incubated for 2 and 4 days at 20 °C on 1/10 MA were observed by scanning electron microscopy to assess the occurrence of resting coccoid cells according to Nishijima *et al.* (2009). Growth was determined in marine broth 2216 (MB; Difco) containing various NaCl concentrations (2, 3, 4, 5, 6, 7, 8, 10, 15 and 20 %, w/v). Growth in NaCl concentrations lower than 2 % (0, 0.5 and 1.0 %) was checked using modified marine broth 2216 which contained the following (l^{-1} distilled water): peptone, 5.0 g; yeast extract, 1.0 g; ferric citrate, 0.1 g; $MgCl_2$, 5.9 g; $MgSO_4$, 3.24 g; $CaCl_2$, 1.8 g; KCl, 0.55 g; KBr, 0.08 g; SrCl, 34.0 mg; boric acid, 22.0 mg; NH_4NO_3, 1.6 mg. The pH range for growth was determined at pH 4.0–10.0 (at intervals of 0.5 pH units) in MB and marine salts basal medium according to González *et al.* (1997). The marine salts basal medium (Mikhailov *et al.*, 2006) contained the following (l^{-1} distilled water): NH_4Cl, 1.0 g; K_2HPO_4, 0.044 g; $FeSO_4 . 7H_2O$, 0.028 g; artificial seawater, 500 ml; Tris/HCl (1 M, pH 7.5), 100 ml. The artificial seawater contained the following (l^{-1} distilled water): NaCl, 23.4 g; $MgSO_4 . 7H_2O$, 24.6 g; KCl, 1.5 g; $CaCl_2$, 2.9 g. The temperature range for growth was determined by using incubation temperatures ranging from 4 to 55 °C.

Oxidase activity was determined by assessing the oxidation of 1 % *p*-aminodimethylaniline oxalate. Catalase activity was determined by assessing bubble production in a 3 % (v/v) H_2O_2 solution. Tests to determine the assimilation of single carbon sources were performed using marine salts basal medium. The corresponding filter-sterilized sugar (0.2 %), alcohol (0.2 %), organic acid (0.1 %) or amino acid (0.1 %) was added to liquid medium. Acid production was tested using the modified MOF medium supplemented with 0.5 % sugars or alcohols (Leifson, 1963; Xu *et al.*, 2008). Biochemical and nutritional tests were performed in MA, as described by Mata *et al.* (2002). API 20E, API 20NE and API ZYM tests (all from bioMérieux) were also used to determine physiological and biochemical characteristics according to the manufacturer's instructions, except that the NaCl concentration in all tests was adjusted to 3.0 %. Incubation time in the API ZYM tests was extended to 4 h.

Fatty acid methyl esters obtained from cells of the two novel strains and strains of three related species [*M. salipaludis* (Yoon *et al.*, 2003a), *M. maritimus* (Yoon *et al.*, 2004) and *M. donghaiensis* (Wang *et al.*, 2009)] grown in MA for 3 days at 30 °C were analysed using GC (6890N GC system; AGILENT) according to the instructions of the MIDI Microbial Identification System. Isoprenoid quinones were analysed using reversed-phase HPLC as described previously (Komagata & Suzuki, 1987). Polar lipids were extracted using a chloroform/methanol system and analysed using two-dimensional TLC, as described previously (Kates, 1986). Merck silica gel 60 F_{254} aluminium-backed thin-layer plates were used in TLC analysis. The plate was dotted with sample and subjected to two-dimensional development, with chloroform/methanol/water (65 : 25 : 4, by vol.) as the first solvent and chloroform/methanol/acetic acid/water (85 : 12 : 15 : 4, by vol.) as the second solvent. The TLC plates were sprayed with molybdatophosphoric acid, followed by heating at 150 °C for 3 min. The G+C content of the deoxyribonucleosides was determined using reversed-phase HPLC and was calculated from the ratio of deoxyguanosine and thymidine (Mesbah & Whitman, 1989).

The 16S rRNA gene was amplified and analysed as described previously (Xu *et al.*, 2007). The sequence was compared with closely related sequences of reference organisms from the EMBL-EBI by the program FASTA and the EzTaxon service (Chun *et al.*, 2007). Sequence data were aligned with CLUSTAL W version 1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony methods (Fitch, 1971) with the MEGA4 program package (Tamura *et al.*, 2007) and by the maximum-likelihood method (Felsenstein, 1981) with TreePuzzle version 5.2. Evolutionary distances were calculated according to the algorithm of Kimura's two-parameter model (Kimura, 1980) for the neighbour-joining method.

Cells of strains Y215^T and Y226^T were both rod-shaped, measuring 0.3–0.5 μm in width and 2.5–5.0 μm in length, after 2 days incubation at 30 °C on MA; electron micrographs of negatively stained cells did not reveal any flagella on these rods (Supplementary Fig. S1, available in IJSEM Online). Cells of strain Y215^T and Y226^T were rod-shaped after 2 days incubation at 20 °C on 1/10 MA, but turned ovoid when the incubation time was extended 4 days under the same conditions (Supplementary Fig. S2, available in IJSEM Online); this is similar to the situation observed in other species of the genus *Microbulbifer* (Nishijima *et al.*, 2009).

The two novel strains and the reference *Microbulbifer* species grew at similar pH ranges. However, a comparison of physiological and biochemical characteristics showed differing results for colonial pigmentation, temperature and NaCl concentration for growth, catalase activity, *N*-acetyl-*β*-glucosaminidase activity, hydrolysis of gelatin, starch and Tween 80, sole carbon source utilization and sensitivity to antibiotics. Detailed information regarding phenotypic and biochemical tests is given in the species description or shown in Table 1.

Two almost-complete 16S rRNA gene sequences (1497 nt each) of strains Y215^T and Y226^T were obtained; the sequence similarity between strains Y215^T and Y226^T was 96 %. Both strains had highest 16S rRNA gene sequence similarity to members of the genus *Microbulbifer*. Strain

Table 1. Differential phenotypic characteristics of strains Y215^T and Y226^T and other reference *Microbulbifer* species

Strains: 1, Y215^T; 2, Y226^T; 3, *M. hydrolyticus* ATCC 700072^T (González *et al.*, 1997); 4, *M. salipaludis* JCM 11542^T (Yoon *et al.*, 2003a); 5, *M. maritimus* JCM 12187^T (Yoon *et al.*, 2004); 6, *M. donghaiensis* JCM 15145^T (Wang *et al.*, 2009); 7, *Microbulbifer variabilis* ATCC 700307^T (Nishijima *et al.*, 2009). Unless indicated, data are from this study. +, Positive; −, negative; W, weakly positive; V, variable; ND, no data. *M. salipaludis* JCM 11542^T (bought from JCM), *M. maritimus* JCM 12187^T (bought from JCM) and *M. donghaiensis* JCM 15145^T (provided by the authors) were incubated aerobically for 2 or 3 days at the optimum temperature on MB and stored at −80 °C in 15 % (v/v) glycerol.

Characteristics	1	2	3	4	5	6	7
Morphology	Rods–cocci	Rods–cocci	Rods–cocci[a]*	Rods–cocci[a]	Rods–cocci[a]	Rods[b]	Rods–cocci[a]
Resting coccoid cells	+	+	+[a]	+[a]	+[a]	ND	+[a]
Colony colour	Light yellow	Yellow	Cream[a]	Greyish-yellow	Yellowish-brown	Light yellow	Cream[a]
Growth in 10 % NaCl	−	+	−[a]	+[c]	+[d]	−[b]	−[a]
Growth at pH 5.0	+	+	−[e]	+[c]	+[d]	−[b]	−[a]
Growth temperature (°C)							
Optimum	25–30	30–37	37[e]	37[c]	37[d]	35–37[b]	30[a]
Maximum	40	45	41[e]	45[c]	48[d]	40[b]	42[a]
Catalase activity	−	+	+	+	+	+	+[a]
N-Acetyl-*β*-glucosaminidase	−	−	−	+	−	+	−[a]
Hydrolysis of:							
Gelatin	−	−	+	+	+	−	+[a]
Starch	+	−	−	+	+	+	ND
Tween 80	−	+	+	+	+	+	+[a]
Utilization of:							
L-Arginine	+	+	−	−	+	+	+[a]
Cellobiose	−	+	+	+	+	+	+[a]
Glycine	−	−	−	+	+	−	ND
Lactate	−	−	−	+	−	−	ND
Malate	W	W	+	−	+	+	−[a]
Maltose	+	−	+	+	+	+	V[a]
D-Mannose	−	+	−	−	−	−	V[a]
L-Ornithine	+	+	−	−	+	−	ND
Propionate	+	−	+	+	+	+	+[a]
Rhamnose	−	−	−	+	−	−	ND
L-Serine	+	−	+	+	+	−	−[a]
Sensitive to:							
Ampicillin (10 μg)	−	−	−	+	−	−	ND
Cefotaxime (30 μg)	−	+	+	+	+	+	ND
Cefoxitin (30 μg)	−	+	+	+	+	+	ND
Kanamycin (30 μg)	+	−	−	−	+	+	ND
API ZYM tests							
Cystine arylamidase	−	−	−	−	−	+	−[a]
Valine arylamidase	+	+	−	−	−	+	+[a]
Trypsin	−	W	−	−	−	−	+[a]
DNA G+C content (mol%)	54	56.7	57.7	59[c]	59.9[d]	57.8[b]	48.1–49.7[a]

*Data from: *a*, Nishijima *et al.* (2009); *b*, Wang *et al.* (2009); *c*, Yoon *et al.* (2003a); *d*, Yoon *et al.* (2004); *e*, González *et al.* (1997).

Y215^T was closely related to the type strains of *M. maritimus* (97.6 %) and *M. donghaiensis* (97.5 %), whereas strain Y226^T was closely related to the type strain of *M. salipaludis* (97.6 %). Phylogenetic analysis based on the neighbour-joining method showed that strains Y215^T and Y226^T fell into two separate clusters (Fig. 1). Similar topologies were found in the phylogenetic trees constructed by the maximum-likelihood and maximum-parsimony methods.

The cellular fatty acid compositions of strains Y215^T and Y226^T and the reference *Microbulbifer* species are shown in Supplementary Table S1 (available in IJSEM Online). The major fatty acids (>5 %) were iso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, $C_{18:1}\omega7c$, $C_{16:0}$, iso-$C_{17:0}$ and iso-$C_{11:0}$ 3-OH for strain Y215^T, and iso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, iso-$C_{17:0}$, iso-$C_{11:0}$ 3-OH, iso-$C_{11:0}$ and $C_{18:1}\omega7c$ for strain Y226^T. The fatty acid profiles of the two novel strains and the reference strains were similar, with the exception of *M. salipaludis*, which possessed $C_{18:1}\omega7c$ as the predominant fatty acid (Supplementary Table S1). The predominant isoprenoid quinone detected in strains Y215^T and Y226^T was Q-8, with Q-7 and Q-9 as minor components. The major polar lipids of strains Y215^T and Y226^T were phosphatidylethanolamine, phosphatidylglycerol and an unknown glycolipid (Supplementary Fig. S3, available in IJSEM Online).

DNA–DNA hybridizations were performed using the thermal denaturation and renaturation method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983), using a Beckman DU 800 spectrophotometer. The hybridization temperature used was 75 °C and experiments were carried out in triplicate. The DNA relatedness values of strain Y215^T with *M. maritimus* JCM 12187^T and *M. donghaiensis* JCM 15145^T were 34.1 and 32.8 %, respectively. The DNA relatedness value of strain Y226^T to *M. salipaludis* JCM 11542^T was 38.0 %. All these values are significantly below the value of 70 % considered to be the threshold for delineation of bacterial species (Wayne *et al.*, 1987).

On the basis of the phenotypic, phylogenetic and genotypic data, strains Y215^T and Y226^T represent two novel species within the genus *Microbulbifer*, for which the names *Microbulbifer marinus* sp. nov. (type strain Y215^T) and *Microbulbifer yueqingensis* sp. nov. (type strain Y226^T) are proposed.

Description of *Microbulbifer marinus* sp. nov.

Microbulbifer marinus (ma.ri′nus. L. masc. adj. *marinus* of the sea, marine).

Cells are Gram-negative and non-motile. Colonies on MA are 2.0–3.0 mm in diameter, slightly irregular, convex and light yellow after 3 days at 30 °C. Colonies on 1/10 MA are very thin and spreading. Cells are rod-shaped (0.3–0.5 × 2.5–5.0 μm) in young cultures, but ovoid (1.0–1.7 × 0.6–1.1 μm) in older cultures. Growth occurs at NaCl concentrations of 0–7.0 % (w/v), with optimum growth at 2.0–3.0 %. Grows at pH 4.5–10.0 and 15–40 °C; optimum growth occurs at pH 7.0–8.0 and 25–30 °C. Oxidase-positive but catalase-negative. Nitrate is reduced. Aesculin, casein and starch are hydrolysed. Gelatin, Tween 80 and tyrosine are not hydrolysed. Negative for gluconate oxidation, indole production, *N*-acetyl-β-glucosaminidase, lysine decarboxylase, ONPG hydrolysis, ornithine decarboxylase and urease. H_2S is not produced from thiosulfate. The following substrates are utilized for growth: acetate, L-alanine, L-arginine, glucose, L-glutamate, L-histidine, malate, maltose, L-ornithine, propionate, pyruvate, L-serine, starch, succinate, trehalose and L-valine. The following compounds are not utilized as sole carbon sources: L-arabinose, cellobiose, citrate, L-cysteine, ethanol, formate, D-fructose, fumarate, D-galactose, gluconate, glycerol, glycine, lactate, lactose, malonate, mannitol, D-mannose, L-methionine, raffinose, rhamnose, ribose, sorbitol, sucrose and D-xylose. Acid is produced from glucose and maltose. Acid is not produced from L-arabinose, lactose, raffinose or sorbitol.

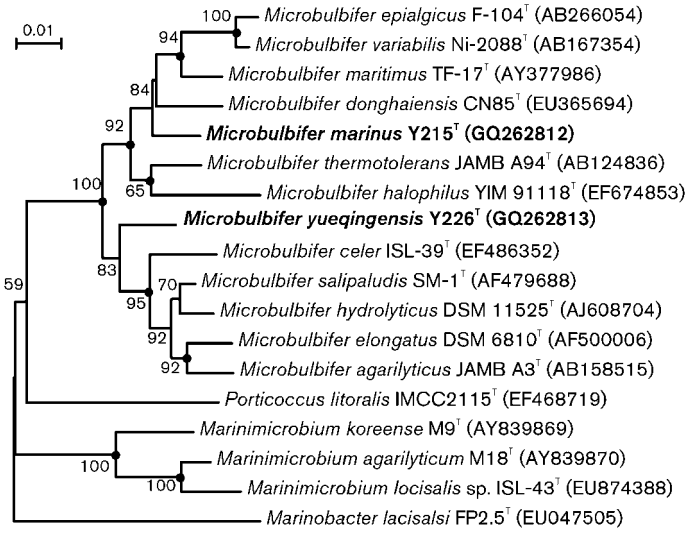

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of the novel isolates and related taxa. Bootstrap values are based on 1000 replicates; only values >50 % are shown. Filled circles indicate that nodes were recovered with bootstrap percentages >50 % in both maximum-likelihood and maximum-parsimony trees. Bar, 0.01 substitutions per nucleotide position.

Susceptible to chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg) and novobiocin (30 μg), but not to ampicillin (10 μg), bacitracin (0.04 IU), cefalexin (30 μg), cefotaxime (30 μg), cefoxitin (30 μg), nitrofurantoin (300 μg), penicillin (10 μg), streptomycin (10 μg) and tetracycline (30 μg). In the API ZYM system, acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase activities are present, whereas *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α- and β-galactosidases, α- and β-glucosidases, β-glucuronidase, lipase (C14), α-mannosidase and trypsin activities are absent. Q-8 is the major respiratory quinone. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and an unknown glycolipid. The major fatty acids (>5 %) are iso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, iso-$C_{17:0}$, iso-$C_{11:0}$ 3-OH, $C_{16:0}$ and $C_{18:1}\omega7c$.

The type strain is Y215^T (=CGMCC 1.10657^T=JCM 17211^T), isolated from a marine sediment sample from Yueqing Bay, Zhejiang Province, China. The DNA G+C content of the type strain is 54.0 mol% (HPLC).

Description of *Microbulbifer yueqingensis* sp. nov.

Microbulbifer yueqingensis (yue.qin.gen′sis. N.L. masc. adj. *yueqingensis* belonging to Yueqing Bay).

Cells are Gram-negative and non-motile. Colonies on MA are 2.0–3.0 mm in diameter, circular, convex and yellow coloured after 3 days at 30 °C. Colonies on 1/10 MA are very thin and spreading. Cells are rod-shaped (0.3–0.5 × 2.5–5.0 μm) in young cultures, but are ovoid (1.0–1.8 × 0.6–1.1 μm) in older cultures. Growth occurs at NaCl concentrations of 0–10.0 % (w/v), with optimum growth at 2.0–3.0 %. Grows at pH 5.0–10.0 and 15–45 °C; optimum growth occurs at pH 7.0–8.0 and 30–37 °C. Oxidase- and catalase-positive. Nitrate is reduced. Aesculin, casein and Tween 80 are hydrolysed. Gelatin, starch and tyrosine are not hydrolysed. Negative for gluconate oxidation, indole production, *N*-acetyl-β-glucosaminidase, lysine decarboxylase, ONPG hydrolysis, ornithine decarboxylase and urease. H_2S is not produced from thiosulfate. The following substrates are utilized for growth: acetate, L-alanine, L-arginine, cellobiose, glucose, L-glutamate, L-histidine, malate, D-mannose, L-ornithine, pyruvate, starch, succinate, trehalose and L-valine. The following compounds are not utilized as sole carbon sources: L-arabinose, citrate, L-cysteine, ethanol, formate, D-fructose, fumarate, D-galactose, gluconate, glycerol, glycine, lactate, lactose, malonate, maltose, mannitol, L-methionine, propionate, raffinose, rhamnose, ribose, L-serine, sorbitol, sucrose and D-xylose. Acid is produced from glucose and maltose. Acid is not produced from L-arabinose, lactose, raffinose or sorbitol. Susceptible to cefotaxime (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), nitrofurantoin (300 μg) and novobiocin (30 μg), but not to ampicillin (10 μg), bacitracin (0.04 IU), cefalexin (30 μg), kanamycin (30 μg), penicillin (10 μg), streptomycin (10 μg) and tetracycline (30 μg). In the API ZYM system, acid and alkaline phosphatases, esterase (C4), esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, valine arylamidase and trypsin activities are present, whereas *N*-acetyl-β-glucosaminidase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α- and β-galactosidases, α- and β-glucosidases, β-glucuronidase, lipase (C14) and α-mannosidase activities are absent. Q-8 is the major respiratory quinone. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and an unknown glycolipid. The major fatty acids (>5 %) are iso-$C_{15:0}$, iso-$C_{11:0}$ 3-OH, iso-$C_{17:1}\omega9c$, iso-$C_{11:0}$, iso-$C_{17:0}$ and $C_{18:1}\omega7c$.

The type strain is Y226^T (=CGMCC 1.10658^T=JCM 17212^T), isolated from a marine sediment sample from Yueqing Bay, Zhejiang Province, China. The DNA G+C content of the type strain is 56.7 mol% (HPLC).

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