Rhizobium marinum sp. nov., a malachite-green-tolerant bacterium isolated from seawater

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A motile, Gram-stain-negative, non-pigmented bacterial strain, designated MGL06^T, was isolated from seawater of the South China Sea on selection medium containing 0.1 % (w/v) malachite green. Strain MGL06^T showed highest 16S rRNA gene sequence similarity to *Rhizobium vignae* CCBAU 05176^T (97.2 %), and shared 93.2–96.9 % with the type strains of other recognized *Rhizobium* species. Phylogenetic analyses based on 16S rRNA and housekeeping gene sequences showed that strain MGL06^T belonged to the genus *Rhizobium*. Mean levels of DNA–DNA relatedness between strain MGL06^T and *R. vignae* CCBAU 05176^T, *Rhizobium huautlense* S02^T and *Rhizobium alkalisoli* CCBAU 01393^T were 20±3, 18±2 and 14±3 %, respectively, indicating that strain MGL06^T was distinct from them genetically. Strain MGL06^T did not form nodules on three different legumes, and the *nodD* and *nifH* genes were also not detected by PCR or based on the draft genome sequence. Strain MGL06^T contained Q-10 as the predominant ubiquinone. The major fatty acid was $C_{18:1}\omega7c$/$C_{18:1}\omega6c$ with minor amounts of $C_{19:0}$ cyclo $\omega8c$, $C_{16:0}$ and $C_{18:1}\omega7c$ 11-methyl. Polar lipids of strain MGL06^T included unknown glycolipids, phosphatidylcholine, aminolipid, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, an unknown polar lipid and aminophospholipid. Based on its phenotypic and genotypic data, strain MGL06^T represents a novel species of the genus *Rhizobium*, for which the name *Rhizobium marinum* sp. nov. is proposed. The type strain is MGL06^T (=MCCC 1A00836^T=JCM 30155^T).

The genus *Rhizobium* was first proposed by Frank (1889) and its description was emended by Young *et al.* (2001); at the time of writing, the genus contained 72 recognized species (http://www.bacterio.net/rhizobium.html). Rhizobia are Gram-negative rod-shaped bacteria that induce the formation of root nodules on legumes and fix nitrogen inside the nodules. Most rhizobia strains have been isolated from nodules of leguminous plants (Mnasri *et al.*, 2014). However, some species of the genus *Rhizobium* have been isolated from other sources (Yoon *et al.*, 2010; Zhang *et al.*, 2011; Turdahon *et al.*, 2013) and feature other functions, such as the triazophos-degrading *Rhizobium flavum* (Gu *et al.*, 2014), polycyclic aromatic hydrocarbon-decomposing *Rhizobium petrolearium* (Zhang *et al.*, 2012), aniline-degrading *Rhizobium borbori* (Zhang *et al.*, 2011) and exopolysaccharide-producing *Rhizobium alamii* (Berge *et al.*, 2009). During our survey of malachite-green-degrading bacteria from marine samples collected in the South China Sea (118° 23′ E 21° 03′ N), one *Rhizobium*-like strain, designated MGL06^T, was isolated from a seawater sample collected on 9 June 2013. Although malachite green is normally toxic to micro-organisms (Chen *et al.*, 2010), strain MGL06^T was able to grow well on Difco™ Marine Agar 2216 medium (BD, USA) medium containing 0.1 % (w/v) malachite green. The aim of the present study was to determine the exact taxonomic position of strain MGL06^T by using a polyphasic approach that included determination of phenotypic properties, phylogenetic investigations based on 16S rRNA and seven housekeeping gene sequences (*recA*, *atpD*,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *atpD*, *recA*, *dnaK*, *thrC*, *gyrB*, *glnII* and *rpoB* gene sequences of strain MGL06^T are KJ751545, KJ941332, KM892804, KM892806, KM892805, KM892808, KM892807 and KM892803, respectively.

One supplementary table and four supplementary figures are available with the online Supplementary Material.

glnII, *gyrB*, *rpoB*, *dnaK* and *thrC*), and DNA–DNA relatedness analysis. This is, to our knowledge, the first *Rhizobium* species isolated from the marine environment and can tolerate a high concentration of malachite green.

One millilitre of seawater was inoculated into 2216E broth (5 g peptone and 2 g yeast extract, per litre seawater, pH 7.6–7.8) with 0.1 % (w/v) malachite green and cultured at 28 °C for 2 months. Strain MGL06^T was isolated by means of the standard dilution plating technique under aseptic conditions when the enrichment culture was diluted with sterile seawater, and the mixture was cultured at 28 °C on MA supplemented with 0.1 % (w/v) malachite green. The colonies were picked and purified. The strain was maintained as viable cultures on plates at room temperature and stored as 16 % (v/v) glycerol suspensions at −80 °C. Three type strains (*Rhizobium vignae* CCBAU 05176^T, *Rhizobium huautlense* S02^T and *Rhizobium alkalisoli* CCBAU 01393^T) were used as reference strains for DNA–DNA hybridization, tests of physiology and biochemistry, and fatty acid analysis. These reference strains and their features were compared with strain MGL06^T under the same laboratory conditions (Table 1).

Chromosomal DNA was isolated and purified using the Rapid Bacterial Genomic DNA Isolation kit (Sangon Biotech) according to the manufacturer's instructions. The 16S rRNA gene of strain MGL06^T was amplified by PCR with Ex *Taq* DNA polymerase (Takara) and two universal primers (27F, AGAGTTTGATCCTGGCTCAG; 1492R, GGTTACC TTGTTACGACTT) (Chun & Goodfellow, 1995). The housekeeping gene sequences of strain MGL06^T were manually obtained from its draft genome (Liu *et al.*, 2014). Sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 6

Table 1. Phenotypic characteristics that differentiate strain MGL06^T from its phylogenetic neighbours in the genus *Rhizobium*

Strains: 1, MGL06^T; 2, *R. vignae* CCBAU 01393^T; 3, *R. huautlense* S02^T; 4, *R. alkalisoli* CCBAU 01393^T. All data are from this study. All strains are positive for growth with 1 % (w/v) NaCl, nitrate reduction, hydrolysis of hypoxanthine, β-galactoside and utilization of L-arabinose and D-mannitol. All strains were negative for utilization of *N*-acetylglucosamine, maltose, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid, and susceptibility to penicillin G. In API ZYM tests, all strains were positive for leucine arylamidase and acid phosphatase, but negative for lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-fucosidase and α-mannosidase. +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4
Origin	Surface seawater (China)	Root nodule of *Astragalus dahuricus* (China)	*Sesbania herbacea* (Mexico)	Nodules of legume species (China)
Flagella	Three or more	None	ND	None
pH range for growth	6.0–9.0	4.5–9.0	5.0–9.0	5.0–9.5
Growth at/with:				
40 °C	−	−	+	−
2 % NaCl	+	−	−	−
9 % NaCl	+	−	−	−
Enzyme activity (API ZYM, API 20NE)				
Alkaline phosphatase	+	−	+	−
Esterase (C4)	+	−	−	+
Esterase lipase (C8)	−	W	+	−
Valine arylamidase	−	−	+	−
Cystine arylamidase	+	−	W	−
α-Glucosidase	W	−	−	+
β-Glucosidase	+	−	−	+
Urease	−	+	+	−
Gelatinase	−	+	+	+
Utilization of:				
L-Arginine	−	−	+	−
D-Glucose	−	+	+	+
D-Mannose	+	−	+	+
Potassium gluconate	+	−	−	−
Susceptibility to:				
Chloromycetin (30 μg)	+	−	+	−
Ampicillin (10 μg)	W	−	−	+
Kanamycin (30 μg)	+	−	−	−
Streptomycin (10 μg)	+	−	−	−
DNA G+C content (mol%)	63.0	60.8	56.8	61.8

(Tamura *et al.*, 2013) with distance options according to the Kimura two-parameter model and clustering with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and minimum-evolution (Rzhetsky & Nei, 1992) methods and supported with bootstrap values based on 1000 replications.

The nearly complete 16S rRNA gene sequence (1504 nt) of strain MGL06^T was determined. Sequence similarity was determined using the EzTaxon-e server (Kim *et al.*, 2012). Strain MGL06^T exhibited 16S rRNA gene sequence similarity of 97.2, 97.0 and 96.9 % to *R. vignae* CCBAU 05176^T, *R. huautlense* S02^T and *R. alkalisoli* CCBAU 01393^T, respectively, and of 93.2–96.7 % to the type strains of other recognized species of the genus *Rhizobium*. Strain MGL06^T located in a relatively independent branch within the genus *Rhizobium* (Fig. 1 and Fig. S1, available in the online Supplementary Material) on the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences. The maximum-likelihood and minimum-evolution trees based on 16S rRNA gene sequences have been combined in Fig. 1 and Fig. S1 using filled circles to indicate nodes that were also recovered in the maximum-likelihood and minimum-evolution trees based on the same sequences. According to 16S rRNA gene sequence similarity, the closest neighbours of strain MGL06^T were *R. vignae* CCBAU 05176^T, *R. huautlense* S02^T and *R. alkalisoli* CCBAU 01393^T. On the phylogenetic tree, these strains also located close to strain MGL06^T, supported by a high bootstrap value. Complete coding DNA sequences of *atpD* (1440 bp), *recA* (1053 bp), *dnaK* (1850 bp), *thrC* (1344 bp), *gyrB* (2420 bp), *glnII* (590 bp) and *rpoB* (4086 bp) genes were obtained from the draft genome of MGL06^T. Levels of sequence similarity between the different *Rhizobium* type strains ranged from 87 to 90 % for the *atpD* gene, 88 to 90 % for *recA*, 85 to 89 % for *dnaK*, 79 to 85 % for *thrC*, 80 to 85 % for *gyrB*, 87 to 89 % for *glnII* and 86 to 90 % for *rpoB*. In neighbour-joining trees based on *atpD* and *thrC* gene sequences, strain MGL06^T formed a relatively independent branch, in the neighbour-joining tree based on *recA* gene sequences, strain MGL06^T formed a clade with *Rhizobium etli* CCBAU 85039^T, and in the neighbour-joining tree based on *dnaK* gene sequences, strain MGL06^T formed a clade with *Rhizobium pseudoryzae* J3-127^T and *Rhizobium rubi* LMG 17935^T, all of which were supported by high bootstrap values (>89 %) (Fig. S2). The neighbour-joining trees based on *atpD*, *thrC*, *recA* and *dnaK* gene sequences were not similar to that obtained based on 16S gene sequences, although all show that strain MGL06^T belongs to the genus *Rhizobium* and is different from recognized type strains. Sequence analyses of the 16S rRNA, *recA*, *atpD*, *glnII*, *gyrB*, *rpoB*, *dnaK* and *thrC* genes showed that strain MGL06^T was phylogenetically related to members of the genus *Rhizobium*.

DNA–DNA hybridization is considered a standard method to define bacterial species (Wayne *et al.*, 1987; Graham *et al.*, 1991). DNA–DNA hybridizations were performed with genomic DNA from strain MGL06^T and *R. vignae* CCBAU 05176^T, *R. huautlense* S02^T and *R. alkalisoli* CCBAU 01393^T, using the methods described by Coram & Rawlings (2002) and Tønjum *et al.* (1998). Strain MGL06^T showed low DNA–DNA relatedness to *R. vignae* CCBAU 05176^T, *R. huautlense* S02^T and *R. alkalisoli* CCBAU 01393^T (mean $\pm$ SD: 20 $\pm$ 3, 18 $\pm$ 2 and 14 $\pm$ 3 %, respectively). These values are below the commonly accepted threshold for the phylogenetic definition of different species (Wayne *et al.*, 1987). The G + C content of the genomic DNA of strain MGL06^T was 63.0 mol% as determined from the draft genome sequence (Liu *et al.*, 2014), which is consistent with the range reported for the genus *Rhizobium*.

General laboratory cultivation was performed on Luria–Bertani medium (LB, containing per litre double distilled water: 10.0 g tryptone, 5 g yeast extract and 10 g NaCl; pH 7.0). The Gram reaction, optimal growth temperature, pH and tolerance to NaCl were studied as described by Xu *et al.* (2012). Nodulation tests were performed by investigating the ability of strain MGL06^T to form nodules on three different leguminous species, *Medicago truncatula*, *Phaseolus lunatus* and *Glycine max* (soybean). No nodule formation was observed in three repeated experiments (data not shown). Even when high-dose inoculation of strain MGL06^T was used on the root hairs, no swollen tissues appeared. The oxidase reaction was tested using an oxidase reagent (bioMérieux). Catalase activity was tested using 3 % H_2O_2 solution. Cell motility and morphology were examined using phase-contrast light microscopy (model 50i; Nikon) and transmission electron microscopy (model JEM-1230; JEOL) from cells at the early exponential phase grown at 28 °C for 1 day. Susceptibility to antibiotics was determined on LB medium by performing the disc diffusion test with 26 antibiotics (Oxoid). Other physiological and biochemical tests were performed using the API 20NE and API ZYM systems (bioMérieux). The strain grew well in medium containing 0.5–9.0 % (w/v) NaCl and at pH 6.0–9.0 and 20–35 °C. Optimal growth conditions of 1.0 % (w/v) NaCl, pH 7.0 and 28 °C were thus selected. Strain MGL06^T showed positive results in catalase and oxidase tests. Antibiotic susceptibility patterns showed that strain MGL06^T was highly susceptible to (μg) chloromycetin (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), norfloxacin (10), ofloxacin (5), polymyxin B (30), streptomycin (10), tetracycline (30), carbenicillin (100), cefobid (30), vibramycin (30), cephalexin (30), rocephin (30), furazolidone (15), cefazolin (30) and co-trimoxazole (25) and susceptible to (μg) cephradin (30), erythromycin (15), piperacillin (100) and rifampicin (5). It was resistant to (μg) ampicillin (10) clindamycin (2), oxacillin (1), penicillin G (10) and vancomycin (30). The phenotypic features of strain MGL06^T are given in the species description and Table 1 and the cell morphology is shown in Fig. S3.

Cells were harvested in the late exponential phase of growth on LB at 28 °C. After centrifugation (5000 *g*, 10 min), the supernatant was discarded and the bacterial biomass was lyophilized. The polar lipids were extracted using a

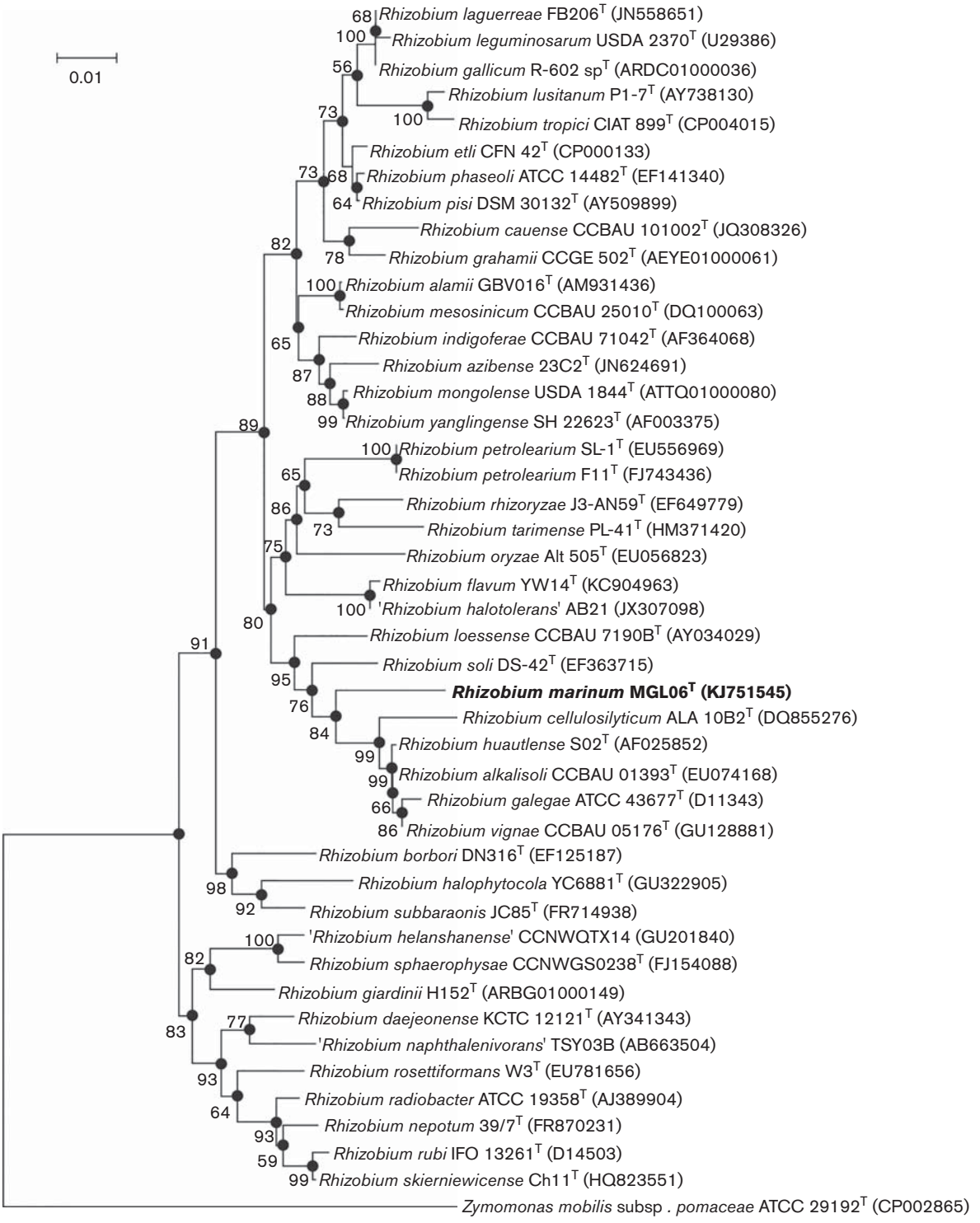

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain MGL06^T among representative members of the genus *Rhizobium*. Filled circles indicate nodes that were also recovered in maximum-likelihood and minimum evolution trees based on the same sequences. *Zymomonas mobilis* subsp. *pomaceae* ATCC 29192^T (CP002865) was used as the outgroup. Bootstrap values above 50 % (based on 1000 replicates) are given at nodes. Bar, 0.01 K_{nuc}.

chloroform/methanol system and separated by two-dimensional TLC using silica gel 60 F254 aluminium-backed thin-layer plates (Merck) (Kates, 1986). The solvent systems chloroform/methanol/water (65 : 24 : 4, by vol.) and chloroform/glacial acetic acid/methanol/water (80 : 12 : 15 : 4, by vol.) were used in the first and second dimensions, respectively. Separated components were visualized by treating the plates with 50 % (w/v) sulfuric acid ethanol solution followed by heating at 120 °C for 10 min. The polar lipids of strain MGL06^T comprised unknown glycolipids (GL1, 2), phosphatidylcholine, aminolipid, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, polar lipid and aminophospholipid (Fig. S4). Polar lipids of strain MGL06^T were consistent with those of related species but with more GL1, phosphatidylethanolamine and diphosphatidylglycerol among recognized species of the genus *Rhizobium*. The major respiratory quinone of strain MGL06^T was ubiquinone 10 (Q10), as determined using reversed-phase HPLC (Komagata & Suzuki, 1987), in line with members of the family *Rhizobiaceae*. Cellular fatty acids were extracted and prepared together with those of the three reference type strains of the genus *Rhizobium* according to the standard protocol of the Microbial Identification System (MIDI; Sherlock). The test was performed using a gas chromatograph (6850; Agilent), and peaks were identified using MIDI software (version 6.0). The major fatty acid of strain MGL06^T was $C_{18:1}\omega7c/C_{18:1}\omega6c$ with minor amounts of $C_{19:0}$ cyclo $\omega8c$, $C_{16:0}$ and $C_{18:1}\omega7c$ 11-methyl. The fatty acid profiles of strain MGL06^T and of the three reference strains were mostly similar, although there were some differences in the proportions of some components (Table S1). Summed feature 8 accounted for a larger proportion of the fatty acids in strain MGL06^T compared with the reference strains. Strain MGL06^T contained $C_{18:0}$ 3-OH and $C_{18:1}\omega7c$ 11-methyl that were not detected in the reference strains.

On the basis of morphological, physiological and chemotaxonomic characteristics, together with 16S rRNA gene sequence analysis, analysis of housekeeping gene sequences and DNA–DNA relatedness data, strain MGL06^T should be assigned to a novel species in the genus *Rhizobium*, for which the name *Rhizobium marinum* sp. nov. is proposed.

Description of *Rhizobium marinum* sp. nov.

Rhizobium marinum (ma.ri′num. L. neut. adj. *marinum* of the sea, marine).

Cells are Gram-reaction-negative, aerobic, non-spore-forming rods (0.61–0.77 μm wide and 1.15–1.88 μm long) that are motile by flagella. On LB, colonies are regular circular, convex with entire margins, smooth and opaque. Grows with 0.5–9.0 % NaCl (optimum 1 %), at 20–35 °C (optimum 28 °C) and at pH 6.0–9.0 (optimum pH 7.0). Nodulation is not observed on three different legumes and the *nodD* and *nifH* genes are not detected by PCR or based on the draft genome sequence. Positive for catalase but negative for oxidase. Nitrate is reduced and reduction of methyl blue and Nile blue is observed. The major fatty acid is $C_{18:1}\omega7c/C_{18:1}\omega6c$ with minor amounts of $C_{19:0}$ cyclo $\omega8c$, $C_{16:0}$ and $C_{18:1}\omega7c$ 11-methyl. In API ZYM tests, positive for alkaline phosphatase, esterase (C4), leucine aminopeptidase, cystine aminopeptidase, acid phosphatase, naphthol-AS-BI-phosphoamidase and α-glucosidase, weakly positive for α-glucosidase and valine aminopeptidase, but negative for β-galactosidase, lipase (C14), esterase lipase (C8), trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. In API 20NE tests, positive for β-glucosidase (aesculin hydrolysis), β-galactoside, and utilization of L-arabinose, D-mannose, D-mannitol, maltose and potassium gluconate; negative for indole production, arginine dihydrolase, urease, gelatin hydrolysis, and utilization of D-glucose, malic acid, *N*-acetylglucosamine, capric acid, adipic acid, trisodium citrate and phenylacetic acid.

The type strain, MGL06^T (=MCCC 1A00836^T=JCM 30155^T), was isolated from a surface seawater sample collected in the South China Sea. The DNA G+C content of the type strain is 63.0 mol%.

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