

Maripseudobacter aurantiacus gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from a sedimentation basin

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Abstract

A Gram-stain negative, orange-pigmented, aerobic, non-motile and ovoid- to rod-shaped bacterial strain, designated CDA4^T, was isolated from a sediment sample collected from the sedimentation basin of a mariculture farm in Zhejiang province, China. The temperature range for growth of strain CDA4^T was 15–40 °C, with an optimum at 35 °C. The pH range for growth was 6.0–8.5, with an optimum around pH 7.5. NaCl was required for growth at the concentration range 0.5–5.0 % (w/v), with an optimum at 2.0 % (w/v). Chemotaxonomic analysis indicated that the main respiratory quinone was menaquinone 6 (MK-6), the predominant cellular fatty acids were iso-C_{15:0}, iso-C_{15:1} G, unknown ECL 13.565 and iso-C_{17:0} 3-OH, and the major polar lipids were phosphatidylethanolamine, two unidentified lipids, two unidentified phospholipids and four unidentified aminolipids. The genomic DNA G+C content of strain CDA4^T was 38.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CDA4^T formed a distinct lineage in the clade of the family *Flavobacteriaceae*. Based on the polyphasic taxonomic characterization, strain CDA4^T is considered to represent a novel species of a new genus, for which the name *Maripseudobacter aurantiacus* gen. nov., sp. nov. is proposed. The type strain of the type species is CDA4^T (=KCTC 52409^T=MCCC 1K03210^T).

The family *Flavobacteriaceae*, belonging to the phylum *Bacteroidetes*, was proposed in 1992 by Reichenbach *et al.* [1] and emended in 2002 by Bernardet *et al.* [2, 3]. At the time of writing, over 150 genera have been described (www.bacterio.net/flavobacterium.html). In general, species of the family *Flavobacteriaceae* are Gram-stain-negative, rod-shaped, chemoheterotrophic, non-pigmented or pigmented (carotenoid or flexirubin pigments or both) and contain menaquinone 6 (MK-6) as the sole respiratory quinone [4–6]. A large number of strains belonging to this family have been isolated from different marine environments, including sediments [7], seawater [8] and marine algae [9]. And members of the family *Flavobacteriaceae* make an important contribution to the remineralization processes in the world's oceans by decomposing bio-organic molecules [10, 11]. Most strains of the family *Flavobacteriaceae* have a low genomic DNA G+C content in the range 30–41 mol% and grow well at 20–30 °C. In this study, we describe the polyphasic characterization of bacterial strain CDA4^T that included determination of chemotaxonomic and phenotypic properties and a detailed

phylogenetic investigation based on 16S rRNA gene sequences. Based on the present study, strain CDA4^T is considered to represent a novel species of a new genus belonging to the family *Flavobacteriaceae*.

Sediment samples were collected from the sedimentation basin of a mariculture farm in Dengbu island (29° 52' N 122° 18' E), Zhejiang Province, China, in summer 2015, and were stored at 4 °C until used. Samples were suspended and diluted by sterile water, using a tenfold dilution series method, and then spread onto modified ZoBell 2216E agar plates [12], which were incubated at 28 °C. The modified ZoBell 2216E medium contained (per litre distilled water): yeast extract 0.5 g, peptone 0.1 g, ferric citrate 0.1 g, NaCl 19.45 g, MgCl₂·6H₂O 8.8 g, CaCl₂·2H₂O 1.8 g, KCl 0.55 g, NaHCO₃ 0.16 g, Na₂SO₄ 3.24 g, KBr 0.08 g, SrCl₂ 34 mg, H₃BO₄ 22 mg, NaSiO₄ 4 mg, NaF 2.4 mg, NH₄NO₃ 1.6 mg and Na₂HPO₄ 8 mg, pH 7.4 adjusted with NaOH. After 72 h of incubation, more than 20 colonies were observed on plates, from which a smooth, orange-coloured colony was picked out and named as CDA4^T. As 16S rRNA gene sequence similarities of lower than 97 % were found with all known

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Keywords: *Flavobacteriaceae*; *Maripseudobacter aurantiacus*.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain CDA4^T is KX364240.

Three supplementary figures and two supplementary tables are available with the online Supplementary Material.

type strains, CDA4^T was purified by streaking on marine agar (MA; BD) and preserved at -80°C in marine broth (MB; BD) supplemented with 20 % (v/v) glycerol for further study.

The 16S rRNA gene of strain CDA4^T was amplified with universal primers 27F and 1492R [13]. PCR products were cloned into the pMD 19T vector (TaKaRa) for sequencing. The almost-complete 16S rRNA gene sequence (1489 nt) was used for pairwise sequence alignment performed by the BLASTN program (www.ncbi.nlm.nih.gov) and the EzTaxon-e server (www.ezbiocloud.net) [14]. Multiple sequence alignment based on 16S rRNA gene sequences of strain CDA4^T and related taxa were performed as described by Zhang *et al.* [15] using MEGA version 5 [16]. As a result, strain CDA4^T was found to be most closely related to species within the family *Flavobacteriaceae*, but shared

sequence similarities of <95.4 % with them. Phylogenetic trees were reconstructed, using *Myroides marinus* JS-08^T as the outgroup, via the neighbour-joining (Fig. 1), maximum-parsimony (Fig. S1, available in the online Supplementary Material) and maximum-likelihood (Fig. S2) algorithms, which all illustrated that strain CDA4^T clustered into the clade of the family *Flavobacteriaceae* by forming a distinct lineage among the most closely related genera: *Maribacter* (92.5–95.3 % 16S rRNA gene sequence similarity), *Zobellia* (92.5–93.8 %), *Pibocella* (93.6 %), *Kriegella* (93.1 %), *Pricia* (92.9 %) and *Arenibacter* (91.9–92.9 %). Based on the phylogenetic analysis, it is suggested that strain CDA4^T could not be assigned to any known genus and should represent a novel species of a new genus in the family *Flavobacteriaceae*. *Zobellia galactanivorans* DSM 12802^T, *Arenibacter latericius* DSM 15913^T and *Maribacter sedimenticola* DSM 19840^T

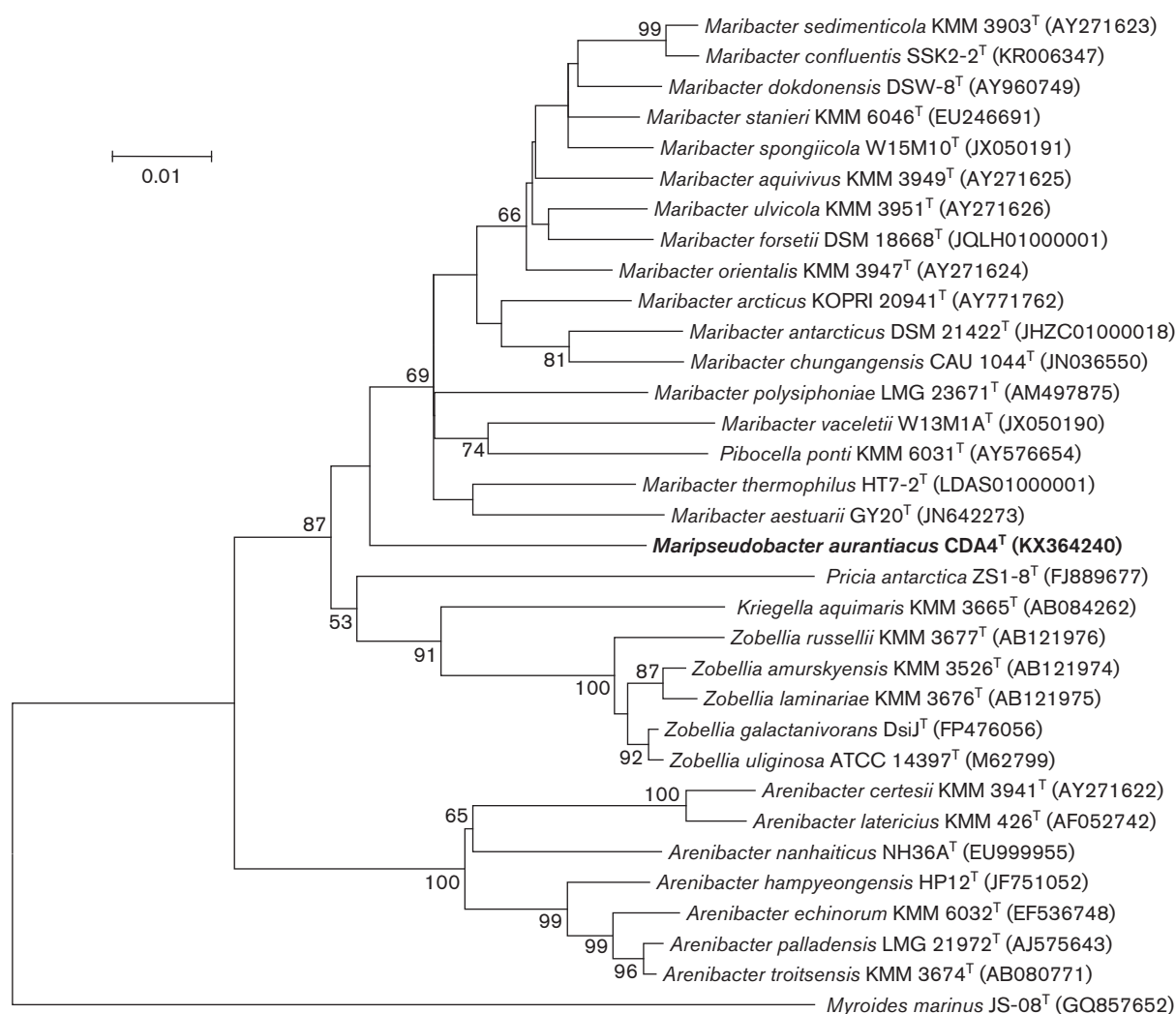


Fig. 1. Neighbour-joining phylogenetic tree based on the 16S rRNA gene sequences of strain CDA4^T and representatives of related taxa. Bootstrap values are expressed as a percentage of 1000 replicates and only those >50 % are given at the branch points. Bar, 0.01 substitutions per nucleotide position.

were used as reference strains. Unless otherwise stated, all strains were incubated on MA or in MB at 28 °C.

The temperature range for growth was tested at 4, 10, 15, 20, 25, 28, 30, 35, 40, 45 and 50 °C in MB. The pH range for growth was determined at 0.5 pH unit intervals by supplementing 30 mM buffering agents in MB at 35 °C, including MES (pH 5.5–6.0), MOPS (pH 6.5–7.5), Tricine (pH 8.0–8.5) and Bis-Tris propane (pH 9.0–9.5). NaCl requirement was measured in MB (pH 7.0) with 0–150 g NaCl l⁻¹, which was increased at a step of 5 g l⁻¹ [17]. Cultures incubated for 3 days were used to determine the optimal growth while those incubated for 14 days were used to determine the growth limits [15]. As a result, growth of strain CDA4^T was observed at 15–40 °C (optimum 35 °C), at pH 6.0–8.0 (optimum pH 7.5) and with 0.5–5 % (w/v) NaCl (optimum 2 %).

Colonies of strain CDA4^T were observed after incubation on MA for 1, 3 and 6 days. Cell morphology was examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL), for which fresh cell suspension was spread on the copper grids, stained with uranyl acetate (0.5 %, w/v) and observed at 80 kV. As shown in Fig. 2, cells of strain CDA4^T were rod-shaped in the exponential growth phase. However, with increased cultivation time, cells became spherical. At the end of cultivation (6–8 days), almost all cells became cocci.

The Gram reaction was tested by using the Gram staining method as described by Dong and Cai [18]. Degradation of starch and L-tyrosine and hydrolysis of Tweens 20, 40, 60 and 80 were tested as described previously [19]. Hydrolysis of hypoxanthine and xanthine was tested as described by Sun *et al.* [19]. Nitrate reduction, urease activity and the ability to hydrolyse casein, chitin, CM-cellulose, filter paper and gelatin were determined according to Dong and Cai [18]. H₂S production, and methyl red and Voges–Proskauer reactions were determined as described previously [20]. Degradation of tyrosine was measured on MA with 5 g tyrosine l⁻¹. The presence of flexirubin-type pigments was investigated using 20 % (w/v) KOH solution [1]. Utilization

of carbon substrates was tested at a concentration of 0.5 % (w/v) according to the protocol of Dong and Cai [18] using whole components of soluble material of MB. Yeast extract (0.01 %, w/v) was added as a growth factor. Anaerobic growth was determined with an AnaeroPack-MicroAero, 2.5l; MGC) anaerobic system using MA, to which 20 mM sodium thiosulfate, 5 mM sodium sulfite, 20 mM sodium sulfate, 5 mM sodium nitrite and 20 mM sodium nitrate were respectively added as electron acceptors [5]. Enzyme activities, acid production, and other physiological and biochemical traits were tested using API ZYM, API 20NE and API 50CH systems (bioMérieux) according to the manufacturer's instructions. Detailed results are described in the genus and species descriptions and in Table S1 [4, 21–25].

Cells used for chemotaxonomic analysis were incubated in MB at 28 °C and 140 r.p.m. for 2 days. Isoprenoid quinones were extracted and purified by TLC, and then identified via an HPLC-MS system (Agilent) [26]. Whole cell fatty acids were analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID) with the standard MIS Library Generation Software version 4.5. Polar lipids were extracted as described by Kates [27] and separated by two-dimensional TLC on silica gel 60 F254 plates (Merck) as recommended by Tindall [28]. Four kinds of spray reagents were used to visualize the corresponding lipids, including molybdophosphoric acid for total lipids, α -naphthol/H₂SO₄ for glycolipids, molybdenum blue for phospholipids and ninhydrin for aminolipids. According to the test results, MK-6 was the predominant quinone (>99 %), which is a common characteristic of the family *Flavobacteriaceae* [29]. The detailed fatty acid profiles of strain CDA4^T and the reference strains are shown in Table S2. The major fatty acids (>10 %) detected in strain CDA4^T were iso-C_{15:0} (45.2 %), iso-C_{15:0} G (15.7 %), unknown ECL 13.565 (11.2 %) and iso-C_{17:0} 3-OH (10.1 %). The fatty acid profiles obtained in this study for the reference strains were similar to those given in the original descriptions [21–23] in terms of the major components, despite some differences in their

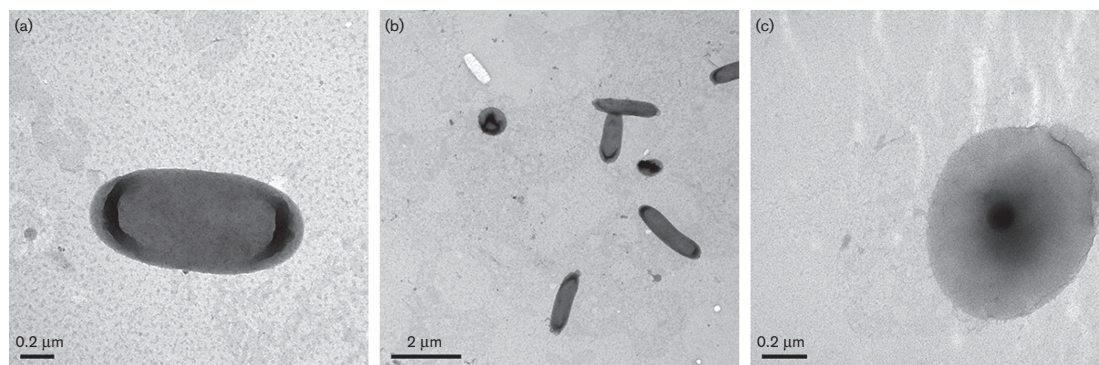


Fig. 2. Transmission electron micrographs of cells of strain CDA4^T. (a–c) Morphology of cells incubated on MA plates at 35 °C for 1, 3 and 6 days, respectively. Bars, (a, c) 0.2 μm; (b) 2 μm.

proportions. The main polar lipids of strain CDA4^T were phosphatidylethanolamine, two unidentified lipids, two unidentified phospholipids and four unidentified aminolipids (Fig. S3).

Genomic DNA was extracted using a bacterial genomic kit (D3350-1; Omega Bio-Tek). The DNA G+C content was determined by reversed-phase HPLC [30] using the genomic DNA of *Escherichia coli* K-12 and salmon sperm DNA (Sigma) as calibration standards. The DNA G+C content of strain CDA4^T was 38.3 mol%, which was similar to those of the genera *Arenibacter* (37–40 mol%), *Zobellia* (36–44 mol%) and *Maribacter* (35–42 mol%), higher than *Pibocella* (35.5 mol%), but lower than *Pricia* (43.9 mol%) and *Kriegella* (39–41 mol%) (Table 1).

Based on the data obtained in this study, strain CDA4^T exhibits the typical characteristics of the family *Flavobacteriaceae*, such as requirement of oxygen, having MK-6 as the major quinone and phosphatidylethanolamine as the predominant polar lipid. However, there are also a number of differences between strain CDA4^T and the most closely

related *Flavobacteriaceae* genera; for example, the optimum temperature for growth of strain CDA4^T (35 °C) is higher than for most members of related taxa, no growth occurs below 15 °C or at >5 % (w/v) NaCl, and the strain is oxidase-negative (Table 1). These are the most conspicuous features of strain CDA4^T and differ from all members of species of related genera. In addition, strain CDA4^T shows variability in cell morphology from rods to cocci, and colonies are orange-pigmented. The strain grows well above 40 °C, higher than the range for growth of *Arenibacter* (5–37 °C), *Pricia* (0–25 °C) and *Kriegella* (4–37 °C). Strain CDA4^T also has some distinctive physiological and biochemical characteristics, including: no H₂S production from Na₂S₂O₃; esterase (C4), valine arylamidase, cysteine arylamidase and trypsin activity; hydrolysis of Tween 60, utilization of melibiose and maltose, acid production from methyl β-D-xylopyranoside, D-galactose and L-rhamnose, and no utilization of D-fructose, L-arabinose, D-lactose or D-mannose (Table S1). As shown in Table 1, strain CDA4^T had iso-C_{15:0} (45.2 %) as the predominant fatty acid, this fatty acid being present at 6.4–17.3 % in *Arenibacter*, 16.8–23.3 %

Table 1. Differential phenotypic characteristics between strain CDA4^T and closely related members of the family *Flavobacteriaceae*

Taxa: 1, strain CDA4^T; 2, *Arenibacter* (data from seven species, Ivanova et al. [22]; Nedashkovskaya et al. [31–34]; Jeong et al. [35]; Sun et al. [36]; this study); 3, *Zobellia* (data from five species, Nedashkovskaya et al. [37]; Barbeyron et al. [23]); 4, *Maribacter* (data from fourteen species, Zhang et al. [38]; Jackson et al. [39]; Cho et al. [40]; Nedashkovskaya et al. [21, 41, 42]; Barbeyron et al. [43]; Hu et al. [44]; Tang et al. [45]; Yoon et al. [46]; Weerawongwiwat et al. [47]; Park et al. [48]; Lo et al. [49]; this study); 5, *Pibocella* (data from Nedashkovskaya et al. [24]); 6, *Pricia* (data from Yu et al. [4]); 7, *Kriegella* (data from Nedashkovskaya et al. [25]). All the taxa are positive for catalase and production of acid from carbohydrates, have MK-6 as the major respiratory quinone and require Na⁺ or seawater for growth. +, Positive; –, negative; v, variable; NA, not available for all species; (NA), not available for certain species.

Characteristic	1	2	3	4	5	6	7
Flexirubin-type pigment	–	–	+	–	–	–	+
Growth above 40 °C	+	–	+	+	+	–	–
Growth below 15 °C	–	+	+	+	+	+	+
Optimum temperature for growth (°C)	35	23–30	21–35	21–24	21–24	17–19	25–28
Salinity range for growth (%)	0.5–5.0	0.5–6	1–10	0–10	1–13	0.5–6	1–6
Oxidase activity	–	+	+	+	+	+	+
Nitrate reduction	–	v	+	v	–	+	–
Degradation of:							
Agar	–	–	+	v	–	–	–
Casein	–	–	+	–	+	–	–
Gelatin	+	v	+	v	+	–	–
Starch	–	–	+	v	+	–	–
Major fatty acids:							
iso-C _{15:0}	45.2	6.4–17.3	16.8–23.3	9.4–36.0	8.7	17.0	12.3
iso-C _{15:1} G*	15.7	9.3–19.2	8.4–14.9	13.8–21.9	11.7	27.2	19.7
Unknown ECL 13.565	11.2	NA	NA	7.9 (NA)	NA	12.3	NA
iso-C _{17:0} 3-OH	10.1	0.3–17.4	14.8–22.4	7.2–31.1	5.6	6.1	12.8
C _{15:0}	2.0	8.2–29.1	7.3–14.4	3.4–14.1 (NA)	4.2	3.6	11.1
Summed feature 3†	2.6	10.3–20.4 (NA)	11.0–15.5	2.8–14.8 (NA)	11.4	15.3	9.4
DNA G+C content (mol%)	38.3	37–40	36–44	35–42	35.5	43.9	39–41

*The position and configuration of the double bond was not known.

†Summed feature 3 (ECL 15.818) is composed of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

in *Zobellia*, 9.4–36.0% in *Maribacter*, 8.7% in *Pibocella ponti* KMM 6031^T, 12.3% in *Kriegella aquimaris* KMM 3665^T and 17% in *Pricia antarctica* ZS1-8^T. In addition, iso-C_{15:0} G (15.7%), unknown ECL 13.565 (11.2%) and iso-C_{17:0} 3-OH (10.1%), as major fatty acids (>10.0% of the total) in strain CDA4^T, show significant differences in component ratios in the reference strains. Most type strains of the genera *Arenibacter*, *Zobellia* and *Maribacter* have C_{15:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) as major fatty acids, but these are minor components in strain CDA4^T (2.0 and 2.6%, respectively). Generally, the content and proportion of predominant and major fatty acids for strain CDA4^T were different from all members of the reference genera.

Based on phenotypic, chemotaxonomic and genotypic characterization, it is concluded that strain CDA4^T is distinct from related genera, including *Arenibacter*, *Zobellia*, *Maribacter*, *Pibocella*, *Pricia* and *Kriegella*, and therefore represents a novel species of a new genus in the family *Flavobacteriaceae*, for which the name *Maripseudobacter aurantiacus* gen. nov., sp. nov. is proposed.

DESCRIPTION OF MARIPSEUDOBACTER GEN. NOV.

Maripseudobacter (Ma.ri.pseu.do.bac'ter. L. neut. n. *mare* the sea; Gr. adj. *pseudēs* false; N.L. masc. n. *bacter* from Gr. n. *baktron* rod; N.L. masc. n. *Maripseudobacter* pseudo-rod inhabiting marine environments).

Cells are rods to cocci, non-spore-forming and obligately aerobic. Gram-stain-negative, catalase-positive and oxidase-negative. Strictly heterotrophic. The predominant menaquinone is MK-6. The major fatty acids (>10% of the total) are iso-C_{15:0}, iso-C_{15:0} G, unknown ECL 13.565 and iso-C_{17:0} 3-OH. The polar lipid profile consists of phosphatidylethanolamine, an unidentified lipid, two unidentified phospholipids and three unidentified aminolipids. A member of the family *Flavobacteriaceae*. The type species is *Maripseudobacter aurantiacus*.

DESCRIPTION OF MARIPSEUDOBACTER AURANTIACUS SP. NOV.

Maripseudobacter aurantiacus (au.ran.ti.a'cus. N.L. masc. adj. *aurantiacus* orange-coloured, referring to the orange colour of the colonies).

Has the following characteristics in addition to those given for the genus. Rod-shaped cells are 0.4–0.7 μm wide and 1.4–3.2 μm long while cocci are 0.4–1.5 μm in diameter. Colonies on MA are circular, smooth, glistening, orange-pigmented and 0.4–1.7 mm in diameter after incubation for 7 days at 35 °C. Growth occurs at 15–40 °C (optimum 35 °C), at pH 6.0–8.0 (optimum pH 7.5) and with 0.5–5.0% (w/v) NaCl (optimum 2%). Sodium ions are required for growth. Positive for hydrolysis of L-tyrosine, gelatin and Tweens 20 and 60. Negative for production of H₂S, flexirubin-type pigments, and hydrolysis of casein, CM-cellulose,

crystalline cellulose (filter paper), starch and Tweens 40 and 80. Glycerin, sodium malonate, pyruvic acid, melibiose, maltose, sucrose, D-glucose, succinic acid, citric acid, sodium acetate and ethanol can serve as the sole carbon source, but not D-ribose, cellobiose, cysteine, fumaric acid, D-fructose, L-arabinose, xylose, α-lactose, L-sorbose, α-ketoglutaric acid, oxalic acid, D-mannose or trehalose.

The type strain is CDA4^T (=KCTC 52409^T=MCCC 1K03210^T), which was isolated from a sediment sample collected from the sedimentation basin of a mariculture farm in Dengbu island, Zhejiang province, China. The DNA G+C content of the type strain is 38.3 mol%.

Funding information

This work was supported by the National Natural Science Foundation of China (grant no. 31470005), the Natural Science Foundation of Zhejiang Province of China (LY14C160008), the Professional Development Program for Teachers in colleges and universities of Zhejiang Province (Screening of marine antagonistic bacteria against the bacterial wilt of *Casuarina* caused by *Ralstonia solanacearum*) and the Talent Startup Project of the Research Development Fund of Zhejiang Agricultural and Forestry University (2014FR074).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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