**Callovatus sediminis** gen. nov., sp. nov., a moderately thermophilic bacterium isolated from a hot spring

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**Abstract**

A Gram-stain-negative, ovoid-shaped, aerobic, non-motile, catalase- and oxidase-positive, and moderately thermophilic bacterial strain, designated strain YIM 72346T, was isolated from a sediment sample collected from a hot spring in Tengchong county, Yunnan province, south-west China. Growth occurred at 37–50 °C (optimum, 45 °C), at pH 6.0–9.0 (optimum, pH 6.5–7.0) and in the presence of 0.5–1.0 % (w/v) NaCl (optimum, 0.5 %). The major cellular fatty acids were C18:1ω7C, C16:0, C19:0 cyclo ω8C, and C18:0 2-OH. The genomic DNA G+C content was determined to be 69.8 mol%. The predominant ubiquinone was Q-10. The polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylincholine, one unidentified aminolipid and two unidentified phospholipids. Bacteriochlorophyll α and carotenoid acids were not detected. Strain YIM 72346T was not observed for the accumulation of poly-β-hydroxybutyrate. The strain shared highest 16S rRNA gene sequence identities with *Crenalkalicoccus roseus* strain YIM 78023T (93.3 %) and *Craurococcus roseus* strain NS130T (92.7 %), but formed a distinct lineage within the family *Acetobacteraceae* in the phylogenetic trees. On the basis of genotypic, phenotypic, chemotaxonomic and phylogenetic analyses, strain YIM 72346T is considered to represent a novel genus and species of the family *Acetobacteraceae*, for which the name *Callovatus sediminis* gen. nov., sp. nov. is proposed. The type strain of *Callovatus sediminis* is YIM 72346T (=KCTC 52714T=CGMCC 1.16330T).

The family *Acetobacteraceae*, proposed by Gillis and De Ley, is taxonomically included in the order *Rhodospirillales* of the class *Alphaproteobacteria* [1]. At the time of writing, the family *Acetobacteraceae* comprises 36 genera (http://www.bacterio.net/acetobacteraceae.html). Members of this family have been described to play a major role in the food industry, including in the production of vinegar [2, 3], alcohol [4, 5], foodstuffs [6, 7] and beverages [8, 9]. Members of this family are characterized as obligately aerobic, and have a respiratory type of metabolism with oxygen as the terminal electron acceptor. Q-10 is the predominant ubiquinone for members of the family, although Q-9 is also found in a few members. Some members of this family are characterized by the presence of bacteriochlorophyll α and carotenoid acids [10–13].

During an investigation exploring thermophilic bacterial diversity, strain YIM 72346T was isolated from sediment collected from the Hamazui (literally translated as ‘frog mouth’) hot spring (pH 7.2, temperature 68 °C, 24.95006 N 98.43830° E), located in Tengchong county, Yunnan province, south-west China. For the isolation, 2 g sediment sample was placed into a flask with 50 ml sterile hot spring water. The flask was kept in a shaker at 45 °C, 200 r.p.m. for 2 h. A sample (1 ml) of the resultant suspension was diluted up to 10−2 dilution. Then, 0.5 ml of the 10−2 dilution suspension was spread on R2A agar medium (0.6 g peptone, 0.6 g yeast extract, 0.6 g glucose, 0.6 g casamino acids, 0.6 g soluble starch, 0.3 g Na-pyruvate, 0.3 g K2HPO4·7H2O, 0.005 g MgSO4·7H2O, 15.0 g agar per litre) plates adjusted to pH 7.0. The plates were incubated at 50 °C for 1 week. Purified strain YIM 72346T was routinely cultured on R2A agar at 50 °C, and was also stored as a glycerol suspension (20 %, w/v) at −80 °C.

Morphological characteristics of strain YIM 72346T were determined by observing the 3-day-old culture grown on R2A agar at 45 °C under a light microscope (BH2; Olympus), a scanning electron microscope (SEM, Quanta 200; FEI) and a transmission electron microscope (JEM-2100; FEI) and a transmission electron microscope (JEM-2100; FEI).

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One supplementary table and two supplementary figures are available with the online Supplementary Material.
JEOL). The sample used for scanning electron microscopy was prepared by taking culture grown on an R2A agar plate, and washing and suspending it in 20 mM phosphate buffer (7.2). The cell suspension was fixed with 2.5 % glutaraldehyde at 4 °C for approximately 1.5 h, dehydrated through a gradient series of ethanol and butanol, and finally dried at the critical point. The dried sample was placed onto a stub-bearing adhesive sputter coated with gold under vacuum and observed under the electron microscope.

Gram-staining was carried out by the standard Gram’s reaction and was confirmed by the KOH lysis test [14]. Staining of intracellular inclusions was done with Sudan black B, according to the protocol of Murray et al. [15]. Growth at different temperatures was tested at 20, 28, 37, 45, 50, 55, 60, 65 and 70 °C in the R2A broth medium. Salt tolerance for growth was observed with 0.5–5 % (w/v) NaCl in R2A broth medium (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 %, w/v, respectively). The pH range (4.0–10.0, at intervals of 1.0 pH unit) for growth was tested in R2A broth medium using the buffer system described by Xu et al. [16]. Single-carbon source assimilation tests were performed in a minimal medium composed of Degryse basal salts [17] to which filtered-sterilized yeast extract (0.1 g l⁻¹) was added. The cell suspension was fixed with 2.5 % glutaraldehyde (0.1 g l⁻¹), ammonium sulfate (0.5 g l⁻¹) and the carbon source (2.0 g l⁻¹) were added. Growth of the strain on single carbon sources was examined by measuring the turbidity of cultures incubated at 45 °C in 20 ml screw-capped tubes containing 10 ml medium for up to 14 days. Nitrogen source utilization was observed in a basal liquid medium according to Nie et al. [18]. Determination of oxidase activity was carried out using 1 % (w/v) tetramethyl-p-phenylenediamine as described by Kovacs [19]. Catalase activity was tested using 3 % (w/v) H₂O₂ by assessing bubble production as the positive result. H₂S production, nitrate reduction and hydrolysis of gelatin, casein, starch and cellulose activities were observed as previously described [20, 21]. API strips (API 20NE and API ZYM) were used to determine metabolic properties and some enzyme activities according to the instructions of the manufacturer (BioMérieux).

The organism was found to be Gram-stain-negative, aerobic, non-motile and had ovoid-shaped cells, measuring 0.5–0.6 × 1.2–1.4 µm in diameter (Fig. S1, available in the online Supplementary Material). The organism did not accumulate poly-β-hydroxybutyrate. The isolate grew at 37–50 °C and pH 6.0–9.0 with optimal growth observed at 45 °C and pH 6.5–7.0. Growth of the organism in the presence of 0.5–1.0 % (w/v) NaCl was observed. The isolate was positive for oxidase and catalase activity, and the nitrate reduction test, but negative for H₂S production. The organism could hydrolyse gelatin, starch and aesculin, but not cellulose or casein. Phenotypic properties useful for distinguishing the novel isolate from the closely related strains Crenalkalicoccus roseus YIM 78023T and Craurococcus roseus NS130T are given in Table 1.

Biomass used for chemical studies was obtained from cultures grown on R2A agar plates for 3 days at 45 °C. Polar lipids were extracted and then separated by using two-dimensional thin-layer chromatography and identified using previously described procedures [22, 23]. Cellular fatty acids were extracted, methylated and analysed by using the Microbial Identification System (Sherlock Version 6.1: MIDI database: TSBA6) [24]. Quinones were extracted as described by Collins et al. [25] and analysed by high-performance liquid chromatography (HPLC) [26]. The genomic DNA G+C content was determined by HPLC after enzymatic degradation [27] using E. coli strain DH5αr as the reference. Bacteriochlorophyll and carotenoid acids were extracted with acetone/methanol (7:3, v/v), as described by Yurko et al. [11] and Boldareva et al. [28], and absorption spectra were recorded spectrophotometrically.

The polar lipids of the novel isolate consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, one unidentified aminolipid and two unidentified phospholipids (Fig. S2). The major cellular fatty acids (>5 %) detected for strain YIM 72346T were C₁₈:1ω7c (32.5 %), C₁₆:0 (30.8 %), C₁₉:0 cyc1ω8c (20.7 %) and C₁₈:1ω7c (6.5 %). The detailed fatty acid profile of strain YIM 72346T is given in Table S1. The major respiratory quinone was determined to be Q-10. The genomic DNA G+C content of strain YIM 72346T was 69.8 mol%.

No characteristic peaks indicating the presence of bacteriochlorophyll a and carotenoid acids were detected in the absorption spectra of the whole-cell extracts.

Extraction of genomic DNA and PCR amplification of the 16S rRNA gene sequence were performed as described by Cui et al. [29] and Li et al. [30]. The resulting 16S rRNA gene sequence was compared with available 16S rRNA gene sequences of cultured species from GenBank via the BLAST program and from the EzBioCloud server databases (www.ezbiocloud.net/identify; [31]). Phylogenetic trees were generated with three tree-making algorithms, neighbour-joining [32], maximum-likelihood [33] and maximum-parsimony [34], using the MEGA version 5.0 software package [35]. Kimura’s two-parameter model was used to calculate evolutionary distance matrices of the neighbour-joining method and the maximum-likelihood method [36]. The topology of the phylogenetic trees was evaluated by using the bootstrap resampling method of Felsenstein [37] with 1000 resamplings.

The novel isolate formed a distinct lineage in the family Acetobacteraceae (Fig. 1), shared highest sequence similarities with Crenalkalicoccus roseus YIM 78023T (93.3 %) and Craurococcus roseus NS130T (92.7 %), and was found to cluster with above genera. This phylogenetic relationship was also supported in the trees generated with the maximum-parsimony and maximum-likelihood algorithms.

The organism could be easily differentiated from its closest phylogenetic relatives, Crenalkalicoccus roseus YIM 78023T and Craurococcus roseus NS130, by several phenotypic properties, such as absence of pigmentation, its shape and variance in its ability to utilize a range of carbohydrates as a
sole carbon source (Table 1). Apart from its phenotypic characteristics, the chemotaxonomic properties of the novel isolate also suggested that the strain is quite different from its close relatives. For example, strain YIM 72346T is different from Crenalkalicoccus roseus YIM 78023T and Craurococcus roseus NS130T. The former is slightly thermophilic, but the latter is mesophilic. In the former, the presence of bacteriochlorophyll a and carotenoid acids was not detected and it does not contain carotenoid acids and contains relatively significant levels of fatty acids C16:0 and C19:0 cyclo ω8c (Table S1).

The organism is closely related to Crenalkalicoccus roseus YIM 78023T on the basis of 16S rRNA gene sequence similarity (93.3%). Both organisms are slightly thermophilic and both lack BChl a, but differ in several morphological and chemotaxonomical features. For instance, in the novel isolate YIM 72346T, the lack of carotenoid acids, summed feature 4 and the presence of high amounts of C16:1ω9c, C19:0 cyclo ω8c, and C18:1 2-OH fatty acids, differentiates the organism from Crenalkalicoccus roseus YIM 78023T at the genus level. Concerning the genus Craurococcus, the phylogenetically most closely related isolate, YIM 72346T, is largely distinguished phenotypically and chemotaxonomically from the type strain of Craurococcus roseus NS130T (Table 1). The former is slightly thermophilic, but the latter is mesophilic. In the former, the presence of bacteriochlorophyll a and carotenoid acids was not detected and it does not contain poly-β-hydroxybutyrate, but the latter contains Bacteriochlorophyll I α, carotenoid acids and accumulates poly-β-hydroxybutyrate. Since strain YIM 72346T does not possess the generic characteristics of any of the two closest genera, we therefore suggest a novel genus to accommodate YIM 72346T, for which the name Caldovatus gen. nov. is proposed. Strain YIM 72346T represents the type strain of the type species of this genus, Caldovatus sediminis sp. nov.

**DESCRIPTION OF CALDOVATUS GEN. NOV.**

*Caldovatus* (Cald.o.va’tus. L. adj. caldus hot; L. masc. adj. ovatus egg shaped, ovate. N.L. masc. n. *caldovatus* an ovoid-shaped bacterium living in hot conditions).
Cells are Gram-stain-negative, non-motile, aerobic, ovoid-shaped and non-spore-forming. Colonies are circular, opaque, convex and non-pigmented. Does not contain carotenoids or bacteriochlorophyll α and also does not accumulate poly-β-hydroxybutyrate. Positive for oxidase and catalase activities. The predominant ubiquinone is Q-10. Main cellular fatty acids are (>5 %) C₁₆:0, C₁₉:0 cyclo ω8c, C₁₈:1ω7c and C₁₈:1 2-OH. The polar lipid profile comprises diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, oneaminolipid and two unidentified phospholipids. The genomic DNA G+C content is about 69.8 mol%. The type species is *Caldovatus sediminis*.

**DESCRIPTION OF CALDOVATUS SEDIMINIS SP. NOV.**

*Caldovatus sediminis* (se.di.mi.nis. L. gen. n. *sediminis* of sediment).

The species contains the following characteristics in addition to those listed for the genus.
Cells stain Gram-negative and form non-motile oval-shaped cells that are 0.5–0.6 μm wide and 1.2–1.4 μm long. Colonies are non-pigmented after 3 days of growth on R2A medium at 45°C. Aerobic, oxidase-positive and catalase-positive. Growth occurs at 37–50°C, at pH 6.0–9.0 and with 0.5–1.0% (w/v) NaCl with optimum growth at 45°C, at pH 6.5–7.0 and with 0.5% (w/v) NaCl. Utilizes D-mannose, dulcitol, L-arabinose and sodium malate as the sole carbon sources. Utilizes L-lysine, L-histidine, L-tryptophan and L-tyrosine as nitrogen and energy sources. Activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), valine, tyrosine as nitrogen and energy sources. Utilizes L-arginine, L-glutamine, L-histidine, L-lysine, L-phenylalanine, L-proline, L-tyrosine, L-valine for assimilation of these compounds. Utilizes citrate, malate, succinate, fumarate, lactate, pyruvate, propanoate, butyrate, 2-oxoglutarate, 2-ketobutyrate, ethylenediaminetetraacetic acid (EDTA), 4-nitrophenyl-β-D-galactopyranoside (ONPG) for degradation. Hydrolyses gelatin, aesculin, and starch, but not casein or peptone. Hydrolyzes aesculin, gelatin and starch, but not casein or peptone. 

Hydrolyses aesculin, gelatin and starch, but not casein or peptone. The predominant fatty acids (>5% each) are 16:0, 18:0, 18:1ω6c, 18:1ω7c, 18:2ω6c, 18:2ω7c, 18:3ω3c, 18:3ω6c, and 18:3ω9c. The polar lipid profile comprises diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, one unidentified aminolipid and two unidentified phospholipids. The type strain, YIM 72346T (=KCTC 52714T=CGMCC1.16330T), was isolated from the Hamazui hot spring in Tengchong county, Yunnan province, southwest China. The genomic DNA G+C content is 69.8%. 

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Conflicts of interest

The authors declare that there are no conflicts of interest.

References


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