Terasakiella brassicae sp. nov., isolated from the wastewater of a pickle-processing factory, and emended descriptions of Terasakiella pusilla and the genus Terasakiella

Shuai-Bo Han,1 Yue Su,2 Jing Hu,1 Rui-Jun Wang,2 Cong Sun,1 Dildar Wu,3 Xu-Fen Zhu1 and Min Wu1

Correspondence
Min Wu
wumin@zju.edu.cn
Xu-Fen Zhu
xufenzhu@zju.edu.cn

1College of Life Sciences, Zhejiang University, Hangzhou 310058, PR China
2Ocean College, Zhejiang University, Hangzhou 310058, PR China
3Department of Biology, Xinjiang Normal University, Urumqi 830054, PR China

A Gram-stain-negative, motile, polyhydroxybutyrate-accumulating, aerobic, S-shaped bacterium, designated B3T, was isolated from the wastewater of a pickle-processing factory. 16S rRNA gene sequence similarity analysis showed that it was most closely related to the type strain, Terasakiella pusilla (96.6 % similarity). Strain B3T was able to grow at 4–40 °C (optimum 32–37 °C), pH 5.5–9.0 (optimum 6.5–7.5) and with 0.5–8 % (w/v) NaCl present (optimum 1–2 %, w/v). Chemotaxonomic analysis showed that the respiratory quinone was ubiquinone Q-10, the major fatty acids included C16 : 0, C18 : 1v7c and C16 : 1v7c and/or iso-C15:2-OH. The major polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, aminophospholipid and three uncharacterized phospholipids. The genomic DNA G+C content of strain B3T was 42.3 mol%. The DNA–DNA relatedness value between B3T and T. pusilla DSM 9263T was 23.9 %. On the basis of the phenotypic, chemotaxonomic and genotypic characteristics of strain B3T, it represents a novel species of the genus Terasakiella, for which the name Terasakiella brassicae sp. nov. is proposed. The type strain is B3T (=KCTC 42652T =CGMCC 1.15254T). Emended descriptions of T. pusilla and the genus Terasakiella are also presented.

The genus Terasakiella, belonging to the family Methylocystaceae, was first proposed by Satomi et al. (2002). The type species Terasakiella pusilla was isolated from a putrid infusion of a marine shellfish (Terasaki, 1973). The names 'Spirillum pusillum' (Terasaki, 1973) and Oceanospirillum pusillum (Terasaki, 1979) have been used successively to describe this species. After that, the name was transferred to Terasakiella pusilla based on phylogenetic analysis and in honour of Terasaki by Satomi et al. (2002). At the time of writing, the genus Terasakiella contains only one species with a validly published name, T. pusilla.

In this study, we focus on the description of strain B3T, isolated from the wastewater of a pickle-processing factory in Zhejiang province, PR China. The pH of the wastewater was 7.26 and the salinity was 2.72 % (w/v). T. pusilla DSM 6293T was used as a reference strain. The new isolate represented a novel species of the genus Terasakiella based on the phenotypic and phylogenetic data presented in this study.

The novel isolate was obtained by the following procedure. The wastewater was diluted and spread onto marine agar 2216 (MA) using a tenfold series dilution method. Obvious colonies formed after 2 days’ incubation at 30 °C. Distinctive colonies were picked out and purified by repeated restreaking. Purity was confirmed by the uniformity of cell morphology. The isolate was routinely cultured on marine broth 2216 (MB) medium (BD) and maintained at −80 °C with 20 % (v/v) glycerol.

Cell morphology and motility were determined by using optical microscopy (Olympus; BX40) and transmission electron microscopy (JEOL; JEM-1230) (Huo et al., 2010). Cells grown on plates were suspended and stained.
with uranyl acetate and then fixed on copper mesh before being observed by TEM. Optimal conditions for growth were determined in marine broth 2216 medium containing various NaCl concentrations (0.5 and 1.0–11.0 %, w/v, at increments of 1 %). The temperature range for growth was tested by incubating cells at various temperatures (4, 15, 20, 25, 28, 32, 37, 40, 42, 45 50 °C). The pH range (from pH 5.5 to 10.0, at intervals of 0.5 pH units) was determined in marine broth 2216 medium with the addition of 30 mM buffering agents, including: MES (pH 5.5–6.5), PIPES (pH 6.5–7.5), Tricine buffer (pH 7.5–8.5) and CAPSO (pH 9.0–10.0).

The utilization of single carbon sources was performed with modified BM medium (Farmer & Hickman-Brenner, 2006). The medium contained (l-1 distilled water): 1.0 g NH4Cl, 0.075 g K2HPO4 .7 H2O, 0.028 g FeSO4 .7 H2O, 11.7 g NaCl, 12.3 g MgSO4 .7 H2O, 0.75 g KCl, 1.45 g CaCl2, 10 ml Tris/HCl (10 mM, pH 8.0), and 25 mM PIPES, pH 7.0. API ZYM, 20NE and 50CH kits (Bio Mérieux) were used according to the manufacturers’ instructions. Other biochemical tests were performed using the methods described by Mata et al. (2002). Polyhydroxybutyrate (PHB) production was investigated based on the methods of Zhu et al. (2011). Antibiotic susceptibility tests were determined on MA plates for 3 days at 30 °C using antibiotic discs containing the following (μg per disc, unless indicated): amoxicillin (10), ampicillin (15), bacitracin (0.04 IU), carbencilin (100), cefoxitin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), neomycin (30), norfloxacin (10), novobiocin (30), penicillin G (10 IU), polymyxin (300 IU), rifampicin (5), streptomycin (10), sulfamethoxazole (300) and tetracycline (30). The strains were considered susceptible, intermediate and resistant, respectively, when the diameter of the inhibition zone was >5 mm, 2–5 mm and <2 mm, according to Nokhal & Schlegel (1983).

Cells of strain B3T and T. pusilla DSM 6293T, which were grown on marine broth 2216 medium for 24 h at 30 °C, were used for polar lipid and isoprenoid quinone analysis. Polar lipids were extracted and separated on silica gel 60 F254 aluminium-backed thin-layer plates (10 × 10 cm; Merk 5554) which had dried for 30 min at 55 °C and further analysed according to Minnikin et al. (1984) and Cui et al. (2011). The first dimension of the solvent system was chloroform/methanol/water (65 : 24 : 4, by vol.) and the second dimension was chloroform/glacial acetic acid/methanol/water (80 : 15 : 12 : 4, by vol.). Then the plates were sprayed with sulfuric acid/ethanol (1 : 1, v/v) and heated at 120 °C for 10 min to reveal total lipids. Other reagents such as z-naphthol, ninhydrin and molybdenum blue (Sigma) were used to detect glycolipids, aminolipids and phospholipids, according to Tindall (1990). Phosphomolybdic acid (5 %, w/v, dissolved in alcohol) was sprayed and then heated at 160 °C for 10–15 min to identify total lipids. Ethanol/sulfuric acid/glacial acetic acid/anisaldehyde (135 : 5 : 1.5 : 3.7, by vol.) was also sprayed and heated at 120 °C for 5 min to assist with total polar lipid analysis. Isoprenoid quinones were extracted from freeze-dried cells with chloroform/methanol (2 : 1, v/v) and analysed by reversed-phase HPLC. For the preparation of cellular fatty acid methyl esters (FAMEs), the two strains were harvested and freeze-dried at the exponential stage of growth according to Kuykendall et al. (1988). Identification and quantification of FAMES were performed by the Sherlock Microbial Identification System (MIDI) with standard MIS Library Generation software (Microbial ID).

We used a quick bacteria genomic DNA extraction kit (DongSheng Biotech) to obtain a high quality template. An almost complete 16S rRNA gene sequence of this isolate was obtained by PCR using the primer pair 27F (5′-AGA-GTTTGAATCCTGCGTCAAG-3′) / 1492R (5′-GTTTACCTTGTAGC-3′).
GTACAGCATT-3’) and the PCR products were cloned into a pMD19-T vector (Takara) for sequencing (Xu et al., 2007). The sequence was compared with closely related organisms provided by EzTaxon services (Kim et al., 2012). Multiple sequences were aligned with CLUSTAL W 1.8 (Thompson et al., 1994). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and the maximum-parsimony (Fitch, 1971) methods with the MEGA5 program package. Evolutionary distances were calculated according to the algorithm of Kimura’s two-parameter model (Kimura, 1980) for the neighbour-joining method. The DNA G+C content was determined by reversed-phase HPLC, as described by Mesbah & Whitman (1989). DNA–DNA hybridization experiments were performed with the thermal denaturation and renaturation method according to Zhang et al. (2010), using a Backman DU800 spectrophotometer.

Cells of strain B3T were Gram-stain-negative, PHB-accumulating, S-shaped, helical counter-clockwise (Fig S1, available in the online Supplementary Material) and motile by means of single bipolar flagellum (Fig. 1). Strain B3T grew at 4–40 °C (optimum 32–37 °C), pH 5.5–9.0 (optimum 6.5–7.5) and with 0.5–8 % NaCl (w/v, optimum 1–2 %). Detailed results of physiological and biochemical tests are given in the species description. The differential characteristics between strain B3T and the reference strain are summarized in Table 1.

The polar lipid profile of strain B3T included diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), amino-phospholipid (APL), three uncharacterized phospholipids (PL1–3) and three uncharacterized lipids (L1–3), which differed from that of the reference strain, T. pusilla DSM 6293T, in this study (Fig. S2). The major cellular fatty acids of strain B3T were C16 : 0 (14.5 % of the total), C18 : 1ω7c (28.8 %) and C16 : 1ω7c and/or iso-C15 : 02-OH (33.4 %) (Table 2). Ubiquinone Q-10 was detected as the sole respiratory quinone of strain B3T, which is in accordance with the genus description.

An almost-complete 16S rRNA gene sequence (1461 nt) of strain B3T was obtained. Similarity analysis based on it and those of other representative bacteria with validly published names revealed that the strain belongs to the genus Terasakiella and the most closely strain was T. pusilla DSM 6293T (96.6 %). Phylogenetic analysis, based on 16S rRNA gene sequences, showed that strain B3T clustered with T. pusilla DSM 6293T in the neighbour-joining, maximum-likelihood and maximum-parsimony trees (Fig. 2). The level of DNA–DNA relatedness between strains B3T and T. pusilla DSM 6293T was 23.9 %, lower than the threshold value of 70 % for separating species (Stackebrandt & Goebel, 1994). The DNA G+C content of strain B3T was 42.3 mol% (HPLC).

The phylogenetic analysis, similar cell morphology (S-shaped and bipolar single flagellum), same major fatty

Table 1. Differential phenotypic and genotypic characteristics of strain B3T and T. pusilla DSM 6293T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH range for growth</td>
<td>5.5–9.0</td>
<td>6.0–9.0*</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of: Citrate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Tween 40</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of: Tyrosine</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Enzyme activities (API ZYM): Acid phosphatase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cefoxitin (30 μg)</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Polar lipids: DPG, PG, PE, PS</td>
<td>DPG, PG, PE, APL, PL1, PL2, PL3</td>
<td>APL, PE, PS, APL, PL1, L1, L2, L3, PL3, PL2</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>42.3</td>
<td>45.8</td>
</tr>
</tbody>
</table>

*Data from Terasaki (1979).

Table 2. Fatty acid composition (as a percentage of the total) of strain B3T and T. pusilla DSM 6293T

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12 : 0</td>
<td>5.6</td>
<td>4.8</td>
</tr>
<tr>
<td>C14 : 0</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>C16 : 0</td>
<td>14.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16 : 1ω5c</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>C18 : 1ω7c</td>
<td>28.8</td>
<td>35.5</td>
</tr>
<tr>
<td>Hydroxy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18 : 1 2-OH</td>
<td>–</td>
<td>2.9</td>
</tr>
<tr>
<td>Summed feature 2*</td>
<td>9.6</td>
<td>10.2</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>33.4</td>
<td>26.7</td>
</tr>
</tbody>
</table>

*Summed feature 2 contained iso-C16 : 1ω17c and/or iso-C15 : 0 3-OH. Summed feature 3 contained C16 : 1ω7c and/or iso-C15 : 0 2-OH.
acids (C\(_{16:0}\), C\(_{18:1\alpha7c}\), and C\(_{16:1\alpha7c}\)), major polar lipids and respiratory quinone (Q-10), along with the other characteristics presented in this study classify strain B3\(^T\) in the genus Terasakiella. However, there are some differential characteristics distinguishing strain B3\(^T\) from the reference strain, T. pusilla DSM 6293\(^T\). Firstly, the fatty acids of T. pusilla DSM 6293\(^T\) contained C\(_{18:1\alpha7c}\), but it was not detected in strain B3\(^T\). Secondly, in this study, the polar lipid profiles of the two strains were different. AL, which was presented in T. pusilla DSM 6293\(^T\), was not detected in strain B3\(^T\) and L3 was detected at a similar position. Moreover, B3\(^T\) contained three uncharacterized phospholipids, which is two more than in T. pusilla DSM 6293\(^T\) (PL1, PL2). Thirdly, some biochemical characteristics, such as susceptibility to antibacterial compounds, the activity of acid phosphatase, nitrate reduction, the Voges–Proskauer reaction, the hydrolysis of tyrosine and carbon source utilization patterns differentiated strain B3\(^T\) from T. pusilla DSM 6293\(^T\). Fourthly, the DNA G+C content of strain B3\(^T\) was 3.5 % lower than that of T. pusilla. Finally, the low DNA–DNA relatedness value (23.9 %) between strain B3\(^T\) and T. pusilla DSM 6293\(^T\) clearly indicated that strain B3\(^T\) should represent a novel species of the genus Terasakiella.

Based on the polyphasic taxonomic characterization presented in this study, strain B3\(^T\) is proposed to represent a novel species of genus Terasakiella, with the name Terasakiella brassicae sp. nov.

**Emended description of Terasakiella pusilla**
The major fatty acids include C\(_{16:0}\), C\(_{18:0\alpha7c}\), iso-C\(_{15:0\alpha7c}\) and/or iso-C\(_{15:0\alpha7c}\). The major polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, aminophospholipid and aminolipid. The genomic DNA G+C content varies between 42 and 46 mol%. The rest of

**Emended description of the genus Terasakiella**
Satomi et al. (2002)
Nitrate is reduced to nitrite or not. The major fatty acids include C\(_{16:0}\), C\(_{18:0\alpha7c}\), iso-C\(_{15:0\alpha7c}\) and/or iso-C\(_{15:0\alpha7c}\). The major polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, aminophospholipid and aminolipid. The genomic DNA G+C content is 45.8 mol% (determined by HPLC). Other properties (Terasaki, 1973) are unchanged.
the description is identical to that given by Satomi et al. (2002). The type species is Terasakiella pusilla.

**Description of Terasakiella brassicae sp. nov.**

*Terasakiella brassicae* (bras’i.caes. L. gen. n. brassicae of cabbage, referring to the pickle ingredients, which the type strain was isolated from).

Cells are Gram-stain-negative, polyhydroxybutyrate-accumulating, S-shaped and helically counter-clockwise 1.5 are the predominant cell forms. Cells are approximately 0.3–0.5 μm wide and 1.5–4 μm long, and motile by means of single bipolar flagellum. After incubation for 24 h on marine agar 2216 plates, colonies are 0.5–1 mm in diameter, slightly convex, non-pigmented, smooth and circular. Growth occurs at pH 5.5–9.0, optimum 6.5–7.5. The optimal growth temperature is 32–37 °C and no growth is detected at 4 or 40 °C. The NaCl concentration range for growth is 0.5–8 % (w/v) and optimal growth occurs at 1–2 % (w/v). No growth is observed in anaerobic conditions by anaerobic respiration with S2O32−, SO32−, SO42−, NO2− or NO3− as electron acceptors. Negative for the catalase, methyl red and Voges-Proskauer reactions, nitrate or nitrite reduction, indole production and arginine dihydrolase. No acid is produced from the carbohydrates nitrate or nitrite reduction, indole production and arginine dihydrolase. No acid is produced from the carbohydrates.

The DNA G+C content of the type strain is 42.3 mol% (determined by HPLC).

**Acknowledgements**

This work was supported by grants from the National Natural Science Foundation of China (No. 31470005) and China Ocean Mineral Resources R & D Association (COMRA) Special Foundation (DY125-14-E-02).

**References**


