Pyruvatibacter mobilis gen. nov., sp. nov., a marine bacterium from the culture broth of Picochlorum sp. 122

Guanghua Wang,¹ Mingxing Tang,¹,² Hualian Wu,¹ Shikun Dai,¹ Tao Li,¹ Chenghao Chen,¹,² Hui He,¹ Jiewei Fan,¹ Wenzhou Xiang¹ and Xiang Li¹

¹Key Laboratory of Tropical Marine Bio-resources and Ecology (LMB), Guangdong Key Laboratory of Marine Materia Medica (LMMM-GD), South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, PR China
²University of Chinese Academy of Sciences, Beijing 100049, PR China

A Gram-stain-negative, aerobic bacterium, designated strain GYP-11T, was isolated from the culture broth of a marine microalga, Picochlorum sp. 122. Cells were dimorphic rods; free living cells were motile by means of a single polar flagellum, and star-shaped-aggregate-forming cells were attached with stalks and non-motile. Sodium pyruvate or Tween 20 was required for growth on marine agar 2216. 16S rRNA gene sequence analysis revealed that this isolate shared 94.07 % similarity with its closest type strain, Parvibaculum hydrocarbonaticlasticum EPR92T. Phylogenetic analyses indicated that strain GYP-11T represents a distinct lineage in a robust clade consisting of strain GYP-11T, alphaproteobacterium GMD21A06 and Candidatus Phaeomarinobacter ectocarpi Ec32. This clade was close to the genera Parvibaculum and Tepidicaulis in the order Rhizobiales. Chemotaxonomic and physiological characteristics, including cellular fatty acids and carbon source profiles, also readily distinguished strain GYP-11T from all established genera and species. Thus, it is concluded that strain GYP-11T represents a novel species of a new genus in the order Rhizobiales, for which the name Pyruvatibacter mobilis gen. nov., sp. nov. is proposed. The type strain of Pyruvatibacter mobilis is GYP-11T (=CGMCC 1.15125T=KCTC 42509T).

Cultivation aims to create an artificial system mimicking in situ conditions. Despite extensive studies on ecosystems where sampling is conducted, the conditions used in classical cultivation conditions are often far from the endogenous abiotic and biotic conditions required for microbial growth (Alain & Querellou, 2009). Recently, it has been shown that substrate availability provides a series of ecological niches in which specialized populations can bloom (Sapp et al., 2007; Teeling et al., 2012; Taylor et al., 2014). Thus, addition of particular substrates is essential for the successful cultivation of some uncultured bacteria. The aim of this study was to identify the exact taxonomic standing of strain GYP-11T, a pure culture that requires addition of pyruvate or Tween 20 for growth on marine agar 2216 (MA; BD).

The microalga Picochlorum sp. 122 was isolated from the Indian Ocean, and cultivated outdoors in filtered natural seawater (collected from west of Sanya Bay, China) amended with urea (1 g l⁻¹), NaHCO₃ (1 g l⁻¹), Na₂HPO₄ (8 mg l⁻¹) and FeSO₄·7H₂O (5 mg l⁻¹). The culture broth of Picochlorum sp. 122 was collected at the late exponential phase, stored and transported at room temperature. Strain GYP-11T was initially isolated as a mixture of GYP-11T and GYP-15T (the latter representing a member of another new genus, details of which will be published in due course) from this culture broth by spreading serial dilutions on GYP plates (2 g tryptone, 1 g yeast extract, 3 ml glycerol, 18 g agar powder and 1 litre of aged seawater, pH 8.0; autoclaving at 121 °C for 15 min). Finally, strain GYP-11T was purified by spreading the bacterial mixture on a GYP plate. However, strain GYP-11T was unable to grow in pure culture on GYP or on MA. A substrate screen was performed using the Biolog Gen III Microplate, and the results indicated that carboxylates with at least three carbons could be oxidized. Subsequently, viability of strain GYP-11T was restored by addition of propionate to GYP medium. Finally, pyruvate and Tween 20 were shown to be optimal substrates for growth of strain GYP-11T. Thus, subsequent cultivation aimed to create an artificial system mimicking in situ conditions. Despite extensive studies on ecosystems where sampling is conducted, the conditions used in classical cultivation conditions are often far from the endogenous abiotic and biotic conditions required for microbial growth (Alain & Querellou, 2009). Recently, it has been shown that substrate availability provides a series of ecological niches in which specialized populations can bloom (Sapp et al., 2007; Teeling et al., 2012; Taylor et al., 2014). Thus, addition of particular substrates is essential for the successful cultivation of some uncultured bacteria. The aim of this study was to identify the exact taxonomic standing of strain GYP-11T, a pure culture that requires addition of pyruvate or Tween 20 for growth on marine agar 2216 (MA; BD).

The microalga Picochlorum sp. 122 was isolated from the Indian Ocean, and cultivated outdoors in filtered natural seawater (collected from west of Sanya Bay, China) amended with urea (1 g l⁻¹), NaHCO₃ (1 g l⁻¹), Na₂HPO₄ (8 mg l⁻¹) and FeSO₄·7H₂O (5 mg l⁻¹). The culture broth of Picochlorum sp. 122 was collected at the late exponential phase, stored and transported at room temperature. Strain GYP-11T was initially isolated as a mixture of GYP-11T and GYP-15T (the latter representing a member of another new genus, details of which will be published in due course) from this culture broth by spreading serial dilutions on GYP plates (2 g tryptone, 1 g yeast extract, 3 ml glycerol, 18 g agar powder and 1 litre of aged seawater, pH 8.0; autoclaving at 121 °C for 15 min). Finally, strain GYP-11T was purified by spreading the bacterial mixture on a GYP plate. However, strain GYP-11T was unable to grow in pure culture on GYP or on MA. A substrate screen was performed using the Biolog Gen III Microplate, and the results indicated that carboxylates with at least three carbons could be oxidized. Subsequently, viability of strain GYP-11T was restored by addition of propionate to GYP medium. Finally, pyruvate and Tween 20 were shown to be optimal substrates for growth of strain GYP-11T. Thus, subsequent
growth experiments were performed aerobically on MA or in marine broth 2216 (BD) amended with 0.5 % pyruvate (MAP/MBP) in the dark at 30 °C. Bacterial stocks were stored at −70 °C in sterile aged seawater supplemented with 20 % glycerol (v/v).

The complete 16S rRNA gene sequence of strain GYP-11T was obtained by a genome scan (Illumina Hiseq 2000 sequencing and sequence reconstruction using SOAPdenovo v2.04) at Shanghai Majorbio Bio-pharm Technology. 16S rRNA gene sequence similarities were obtained from the GenBank and EzTaxon-e databases (Kim et al., 2012). Alignment of 16S rRNA gene sequences was performed using the SINA software package (Pruesse et al. 2012) in the SILVA rRNA database. Phylogenetic trees were reconstructed using the maximum-likelihood (Felsenstein, 1981), neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Swofford, 1993) algorithms in the software package MEGA version 5.0 (Tamura et al., 2011). The phylogenetic distance matrices were estimated by the Kimura two-parameter model (Kimura, 1980). The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The complete 16S rRNA gene sequence (1462 bp) of strain GYP-11T was obtained. Results of 16S rRNA gene sequence global alignment in the EzTaxon-e database revealed that strain GYP-11T was related most closely to *Parvibaculum hydrocarboniclasticum* EPR92T with a similarity of 94.07 %. This sequence similarity suggested that strain GYP-11T may represent a novel species of a new genus (Yarza et al., 2014) as the recommended genus threshold is 94.5 %. Phylogenetic analyses based on the maximum-likelihood algorithm indicated that strain GYP-11T represents a distinct lineage in a robust clade (the GYP-11T group) consisting of *Candidatus Phaeomarinobacter ectocarpi* Ec32, strains GYP-11T and alphaproteobacterium GMD21A06 (Fig. 1). This GYP-11T group is close to the genera *Parvibaculum* and *Tepidicaulis* in the order *Rhizobiales* (Fig. 1).
The maximum-likelihood tree topology was also supported by the neighbour-joining and maximum-parsimony algorithms (Figs S1 and S2, available in the online Supplementary Material). However, the bootstrap confidence level between

The maximum-likelihood tree topology was also supported by the neighbour-joining and maximum-parsimony algorithms (Figs S1 and S2, available in the online Supplementary Material). However, the bootstrap confidence level between the GYP-11\textsuperscript{T} group and the Parvibaculum (Schleheck et al., 2004; Lai et al., 2011; Rosario-Passapera et al., 2012) group was ≤50 % in this study (Fig. 1, Figs S1 and S2), so the exact taxonomic standing of the GYP-11\textsuperscript{T} group in the order Rhizobiiales was uncertain. Based on these analyses, Parvibaculum lavamentivorans DS-1\textsuperscript{T} (=MCCC 1A03287\textsuperscript{\text{T}}=DSM 13023\textsuperscript{T}) and Parvularcula bermudensis HTCC 2503\textsuperscript{T} (=MCCC 1A02685\textsuperscript{T}=KCTC 12087\textsuperscript{T}), obtained from the Marine Culture Collection of China (MCCC), were used as reference strains in subsequent studies. These bacteria were grown on MAP or in MBP.

Cellular morphology was determined by transmission electron microscopy (Hitachi TEM System-H7650) and optical microscopy (Olympus BX53). Cell mobility was tested using an optical microscope and the hanging drop technique (Bernardet et al., 2002). The Gram reaction was determined as described by Gerhardt et al. (1994). Catalase activity was determined by observing bubble production in a 5 % (v/v) hydrogen peroxide solution and oxidase activity was determined by using oxidase test strips (Huankai). NaCl requirement and tolerance were tested at 30 °C for 4 days in reconstituted MBP with NaCl concentrations ranging from 0 and 15 % (0, 0.25, 0.5, 1, 2, 3, 5, 6, 8, 10, 13 and 15 %, w/v). Growth at different pH was tested in MBP at 30 °C for 4 days amended with different buffers (0.5 pH unit intervals: pH 5–8, 0.1 M KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4}; pH 8.5–10, 0.1 M NaHCO\textsubscript{3}/Na\textsubscript{2}CO\textsubscript{3}; pH 10.5–11, 0.1 M Na\textsubscript{2}CO\textsubscript{3}/ NaOH). Optimal growth temperature was determined on MAP after 4 days of growth at 4, 10, 15, 22, 28, 37, 40 and 45 °C. The ability to form endospores and hydrolysis of starch, casein, chitin, gelatin and Tweens 20, 40 and 80 were tested as described by Dong & Cai (2001). For alkane degradation, 0.25 % n-dodecane was added to the bacterial culture broth (medium: MB amended with 0.5 % Tween 20); after 7 days of shaking incubation at 30 °C in the dark, the alkane residue was extracted in n-hexane and analysed by using GC (Shimadzu 2014C). Additional carbohydrate metabolism was tested using the API 20NE kit according to the manufacturer’s protocol except that cells were suspended in sterile aged natural seawater. Anaerobic growth was determined by using the nitrate reduction hole of the API 20NE system, which was sealed with mineral oil. Other phenotypic characteristics were tested by following the standard procedures compiled by Tindall et al. (2007).

Cells of strain GYP-11\textsuperscript{T} were Gram-stain-negative, aerobic, non-spore-forming, dimorphic rods (Fig. S3). Free living cells were motile with a single polar flagellum (Fig. S3C), and star-shaped-aggregate-forming cells were attached with stalks and were non-motile (Fig. S3A, B, D). Star-shaped aggregation in marine bacteria has been observed in the genera Rhizobium and Agrobacterium (Rüger & Höfte, 1992). Stalked cells were also observed in Tepidicaulis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2 (3 species)</th>
<th>3 (4 species)</th>
<th>4 (1 species)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimum growth temperature (°C)</td>
<td>30</td>
<td>25–37/ND</td>
<td>28–37</td>
<td>42</td>
</tr>
<tr>
<td>Degradation of:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkane</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alkylbenzenesulfonate</td>
<td>−</td>
<td>+/ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>−</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucosidase</td>
<td>+</td>
<td>V</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Carbon source utilization:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>−</td>
<td>−/ND</td>
<td>V</td>
<td>−</td>
</tr>
<tr>
<td>Malate</td>
<td>w</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Potassium gluconate</td>
<td>w</td>
<td>v</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>−</td>
<td>v</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Acetate</td>
<td>−</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>−</td>
<td>v</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>+</td>
<td>+</td>
<td>V</td>
<td>ND</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+</td>
<td>ND</td>
<td>−/ND</td>
<td>+</td>
</tr>
<tr>
<td>Predominant fatty acid(s)*</td>
<td>SF8 (48 %)</td>
<td>SF8 (39.7–48.7 %) or Cy (35.8 %)</td>
<td>SF8 (52.4–81.9 %) or C\textsubscript{12} : 0 (34.0 %)</td>
<td>SF8 (87.3 %)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>63</td>
<td>61–64</td>
<td>59–66</td>
<td>61</td>
</tr>
<tr>
<td>Quinone(s)</td>
<td>Q-10</td>
<td>Q-10/Q-11</td>
<td>Q-10</td>
<td>Q-10</td>
</tr>
</tbody>
</table>

*SF8: C\textsubscript{18} : 1\textsuperscript{\text{o}7c} and/or C\textsubscript{18} : 1\textsuperscript{\text{c}6c} Cy, cyclo C\textsubscript{19} : 0 \textsuperscript{\text{c}8c}.
Biomass of strain GYP-11T and reference strains for cellular fatty acid analysis was harvested from MAP plates grown at 28 °C at their late exponential phase. The fatty acid composition was analysed by GC (Agilent G6890N) and identified by using the Sherlock Microbial Identification System (Version 6.0) according to the manufacturer's instructions. Respiratory lipoquinones were extracted as described by Collins (1994) and analysed using reversed-phase HPLC (Komagata & Suzuki, 1987). Polar lipids were extracted as described by Kamekura (1993), and identified by spraying with the appropriate detection reagents after two-dimensional TLC (Tindall, 1990). The G+C content of the genomic DNA was determined by using the HPLC method (Mesbah et al., 1989).

The major cellular fatty acids (>10%) of strain GYP-11T were C18:1ω7c/ω6c (48.0%) and C18:ω7c 11-methyl (18.2%) (Table S1). These major cellular fatty acids of strain GYP-11T are similar to those of members of the genera Parvibaculum (Lai et al., 2011; Rosario-Passapera et al., 2012), Tepidicuclus (Takeuchi et al., 2015) and Parvularcula (Arum et al., 2009; Yu et al., 2013; Li et al., 2014). However, members of the genera Parvibaculum, Tepidicuclus and Parvularcula are characterized by cyclo C19:ω08c (12.1–35.8%), C18:ω7c/ω6c (87.3%) and C16:0 (10.0–21.4%), respectively. The respiratory lipoquinone detected was ubiquinone-10 (Q-10), which is in accordance with most of the closely related species (Table 1) except for Parvibaculum lavamentivorans (Q-11; Schleheck et al., 2004) and Parvibaculum indicum (Q-11; Lai et al., 2011). The polar lipids were phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and five amino lipids (Fig. S4). The genomic DNA G+C content of strain GYP-11T was 63 mol%.

In summary, the main properties including aggregation of stalked cells, inability to utilize alkane, unique cellular fatty acid profile and distinct phylogenetic lineage in the order Rhizobiales distinguished strain GYP-11T from all established genera and species. Thus, strain GYP-11T is proposed to represent a novel species of a new genus, for which the name Pyruvatibacter mobilis gen. nov., sp. nov. is proposed.

**Description of Pyruvatibacter mobilis gen. nov., sp. nov.**

Pyruvatibacter (Py.ru.va_ti.bac’ter. N.L. pyruvate; N.L. masc. n. bacter from Gr. n. bakteron rod; N.L. masc. n. Pyruvatibacter pyruvate-using rod).

Cells are Gram-stain-negative, aerobic, non-spore-forming, dimorphic rods. Free living cells are motile by means of a single polar flagellum, and star-shaped-aggregate-forming cells are attached with stalks and non-motile. Catalase- and oxidase-positive. Nitrate reduction is positive. NaCl is required for growth. Tween 20 or pyruvate is required for growth on MA. The respiratory lipoquinone is Q-10. The major fatty acids (>10%) are C18:1ω7c/ω6c and C18:1ω7c 11-methyl. The major polar lipids are phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine. The genomic DNA G+C content is 63 mol%.

The type species is Pyruvatibacter mobilis.

**Description of Pyruvatibacter mobilis sp. nov.**

Pyruvatibacter mobilis (mo’bi.lis. L. masc. adj. mobilis motile).

The description is as for the genus with the following additional properties. Cells are usually 0.22–0.48 μm wide and 1.3–2.45 μm long. Colonies are pale, tiny and opaque on MAP. Growth occurs at pH 6.0–9.0, at 15–45 °C and in the presence of 0.25–8% (w/v) NaCl, and optimally at 30 °C, at pH 7.0–8.0 and with 1–3% NaCl. Gelatin, Tween 20, 40 and 80 are hydrolysed. Production of H2S does not occur. Protease, β-glucosidase and β-galactosidase are positive, while indole production, D-glucose fermentation, arginine dihydrolase and urease are negative. Utilization of adipic acid, propionic acid, sodium butyrate, β-hydroxybutyric acid, D-fructose 6-phosphate, glucuronamide, L-histidine, sodium lactate, acetoacetic acid and pyruvate is positive. Utilization of l-arabinose, D-mannose, D-mannitol, N-acetylgulosamine, maltose, potassium gluconate, malic acid and phenylacetic acid is weakly positive. Utilization of D-glucose, trisodium citrate and capric acid is negative.

The type strain, GYP-11T (=CGMCC 1.15125T =KCTC 42509T), was isolated from the culture broth of a marine microalga, Picochloruma sp. 122.

**Acknowledgements**

This research was supported by the Public Science and Technology Research Funds Projects of Ocean (201305018-3), National Natural Science Foundation of China (Nos. 41206136, 41230962), Guangdong Province and Chinese Academy of Science cooperation Foundation (2012B091100276), Funds for marine renewable energy (GHME2011SW04) and Guangdong Ocean Innovative Demonstration Area of Economic Development Project (SZHY2012-B01-003).

**References**


