Mangroviflexus xiamenensis gen. nov., sp. nov., a member of the family Marinilabiliaceae isolated from mangrove sediment

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A Gram-negative, obligately anaerobic, non-spore-forming, long rod-shaped bacterium strain P2T was isolated from the offshore mangrove sediment of the South China Sea. Growth was observed at between 22 and 39 °C, with an optimum at 35 °C. The pH range for growth was 5.0–8.5, with an optimum around pH 7.0–7.5. Salt tolerance was determined between 0.2 and 3.5 % (w/v), optimum at 0.5–1.0 %. Catalase and oxidase activities were negative. Strain P2T utilized cysteine, lactate, pyruvate, yeast extract or H2/CO2+acetate as electron donors, and sulfate or sulfite as electron acceptors. Metabolism was strictly fermentative. The main organic fermentation products were propionate, acetate and succinate. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain P2T formed a distinct evolutionary lineage within the family Marinilabiliaceae. Strain P2T was most closely related to members of the genera Alkaliflexus (92.0 % 16S rRNA gene sequence similarity), Marinilabilia (91.7 %) and Anaerophaga (89.9 %) of the family Marinilabiliaceae. The DNA G+C content of the novel strain was 44.2 ± 1.0 mol%. The dominant fatty acids of strain P2T were iso-C15:0 (33.5 %), anteiso-C15:0 (18.9 %), C16:0 (5.4 %), C16:0 3-OH (7.7 %) and iso-C17:0 3-OH (13.3 %). The respiratory quinone was menaquinone 7 (100 % of total quinone) and the major polar lipid was phosphatidylethanolamine. Strain P2T was distinguishable from members of phylogenetically related genera by differences in several phenotypic properties. On the basis of phylogenetic, phenotypic and physiological evidence, a novel genus, Mangroviflexus, is proposed to harbour strain P2T (\textsuperscript{5}CGMCC 1.5167\textsuperscript{T} = DSM 24214\textsuperscript{T}) which is described as the type strain of a novel species, Mangroviflexus xiamenensis gen. nov., sp. nov.
was conducted on board immediately after sampling. Strain P2\textsuperscript{T} were isolated by the streak plate method in an anaerobic atmosphere (Forma Anaerobic System, Thermo Electron) on YTBC marine agar medium (per litre of artificial seawater; ASW): yeast extract 1.0 g, tryptone 5.0 g, Casamino acids 5.0 g, beef extract 5.0 g, K\textsubscript{2}HPO\textsubscript{4} 1.0 g, NH\textsubscript{4}NO\textsubscript{3} 1.0 g, Na\textsubscript{2}WO\textsubscript{4}.2H\textsubscript{2}O 0.1 mg, Na\textsubscript{2}SeO\textsubscript{3}.5H\textsubscript{2}O 0.1 mg, NiCl\textsubscript{2}.6H\textsubscript{2}O 7.5 mg, resazurin 1.0 mg, cysteine/HCl 0.5 g and Na\textsubscript{2}S.9H\textsubscript{2}O 0.5 g. ASW contained (per litre of distilled water): NaCl 5 g, MgCl\textsubscript{2}.6H\textsubscript{2}O 0.75 g, MgSO\textsubscript{4}.7H\textsubscript{2}O 1.2 g, NaHCO\textsubscript{3} 0.05 g, CaCl\textsubscript{2}.2H\textsubscript{2}O 0.1 g, KCl 0.1 g and KH\textsubscript{2}PO\textsubscript{4} 0.1 g. The final pH of YTBC medium was adjusted to 7.0–7.5. For morphological and biochemical characterization, strain P2\textsuperscript{T} was also cultivated on YTBC medium.

Cell morphology was examined by using light spectroscopy (Nikon TE2000 S/U) and transmission electron microscopy (JEM-1230; JEOL). Gram staining was carried out according to the method described by Cowan & Steel (1993). Catalase and oxidase activities were determined by using the bioMérieux oxidase reagent kit according to the manufacturer’s instructions. Growth under different conditions was monitored by measuring the optical density at 600 nm. Growth was evaluated at various temperatures (range 4–50 °C) and pH values (range pH 5.0–10.0) adjusted with HCl or NaOH solutions in YTBC broth. Salt requirement and tolerance were also determined in YTBC medium with between 0 and 5 % NaCl (w/v). Nitrite, nitrate, sulfate, sulfite, thiosulfate (20 mM each) and elemental sulfur (20 mg ml\textsuperscript{−1}) were tested as alternative electron acceptors by replacing sulfate in the growth medium. Oxygen was also tested as the electron acceptor in liquid and solid media. Utilization of electron donors by the isolates was determined by using acetate, DL-lactate, pyruvate, Casamino acids, cysteine, yeast extract, succinate, L-malate, citrate, oxalate, formate, propionate, butyrate, methanol and ethanol at a final concentration of 20 mM. Utilization of H\textsubscript{2} as an electron donor was determined in the presence or absence of acetate (5 mM) as an organic carbon source under an H\textsubscript{2}/CO\textsubscript{2} atmosphere. Substrate utilization was tested in anaerobic liquid medium containing one of the following substrates, each at 1 % (w/v): cellobiose, glucose, xylose, arabinose, maltose, manose, mannitol, trehalose, sorbitol, lactose, fructose, starch, rhamnose, sucrose, galactose and glycerol. The fermentation products were measured in culture with a defined amount of cellobiose according to the method described by Zhilina \textit{et al.} (2004). The formation of hydrogen sulfide was tested as described by Cord-Ruwisch (1985). These parameters were evaluated by growing cells in YTBC medium for 48 h under the relevant conditions. These

### Table 1. Characteristics that differentiate strain P2\textsuperscript{T} from related genera of the family \textit{Marinilabiliaceae}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell motility</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>ND</td>
</tr>
<tr>
<td>Spore formation</td>
<td>–</td>
<td>–</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>O\textsubscript{2} metabolism</td>
<td>Strictly anaerobic</td>
<td>Anaerobic; low O\textsubscript{2} tolerance</td>
<td>Facultatively anaerobic</td>
<td>Strictly anaerobic</td>
</tr>
<tr>
<td>Pigment</td>
<td>Flexirubin</td>
<td>Carotenoid</td>
<td>Carotenoid + flexirubin</td>
<td></td>
</tr>
<tr>
<td>Size (mm)</td>
<td>0.25 × 6.0–6.5</td>
<td>0.25–0.4 × 4–10</td>
<td>0.3–0.5 × 2–50</td>
<td>0.3 × 3–8</td>
</tr>
<tr>
<td>Colour</td>
<td>Light yellow</td>
<td>Pink</td>
<td>Yellow to salmon</td>
<td>Orange–red</td>
</tr>
<tr>
<td>Growth conditions (optimal)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl (%)</td>
<td>0.2–3.5 (0.5–1.0)</td>
<td>0.08–5.5 (2.0)</td>
<td>1.0–3.0</td>
<td>2.0–12.0 (2.0–6.0)</td>
</tr>
<tr>
<td>pH values</td>
<td>5.0–8.5 (7.0–7.5)</td>
<td>7.2–10.2 (8.5)</td>
<td>(7.0–7.5)</td>
<td>(6.8)</td>
</tr>
<tr>
<td>Catalase</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Substrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+/−</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Trehalose</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fermentation products</td>
<td>Propionate, acetate, succinate, H\textsubscript{2}S</td>
<td>Propionate, acetate, succinate, formate</td>
<td>Formate, acetate, propionate, lactate, succinate, H\textsubscript{2}</td>
<td>Propionate, acetate, succinate</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>44.2</td>
<td>44.3</td>
<td>37.0</td>
<td>41.8</td>
</tr>
<tr>
<td>Source</td>
<td>Mangrove</td>
<td>Soda lake</td>
<td>Marine</td>
<td>Oilfield</td>
</tr>
</tbody>
</table>

Taxa: 1, \textit{Mangroviflexus xiamenensis} gen. nov., sp. nov. P2\textsuperscript{T}; 2, \textit{Alkaliflexus imshenetskii} Z-7010\textsuperscript{T} (Zhilina \textit{et al.}, 2004); 3, \textit{Marinilabilia salmonicolor} Cys1\textsuperscript{T} (Nakagawa & Yamasato, 1996); 4, \textit{Anaerophaga thermohalophila} Fru22\textsuperscript{T} (Denger \textit{et al.}, 2002; Holmes \textit{et al.}, 2007). ND, No data. +, Growth observed; −, growth not observed; +/−, growth was weak.
Genomic DNA was prepared according to the method of Ausubel et al. (1995) and the 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R (Weisburg et al., 1991). Sequences of related strains were obtained and analysed by using BLAST searches against the GenBank and EzTaxon databases (Altschul et al., 1997; Chun et al., 2007). The 16S rRNA gene sequences of closely related taxa obtained from the GenBank database were aligned using CLUSTAL_X (Thompson et al., 1997). Clustering with the neighbour-joining (Saitou & Nei, 1987) method was determined by using bootstrap values based on 1000 replications. A tree constructed using the neighbour-joining method is shown in Fig. 1. A nearly full-length 16S rRNA gene sequence (1440 nt) of strain P2\textsuperscript{T} was determined. Sequence analysis of the 16S rRNA gene of strain P2\textsuperscript{T} showed an affiliation with the phylum Bacteroidetes. A phylogenetic tree showed that strain P2\textsuperscript{T} formed a distinct evolutionary lineage within the family Marinilabiliaceae and placed it in a phylogenetic position most closely related to the genus *Alkaliflexus* (Fig. 1). The novel isolates and the genera *Alkaliflexus*, *Marinilabilia* and *Anaerophaga* constituted a phylogenetic cluster with a high bootstrap value. The closest relatives were *Alkaliflexus* imshenetskii Z-7010\textsuperscript{T}, *Marinilabilia salmonicolor* Cys1\textsuperscript{T} and *Anaerophaga* thermohalophila Fru22\textsuperscript{T}, with sequence similarities of 92.0, 91.7 and 89.9\%, respectively, to P2\textsuperscript{T}. All of the 16S rRNA gene sequence divergences between strain P2\textsuperscript{T} and recognized species were greater than 8.0\% and the distinct phylogenetic relationships revealed that strain P2\textsuperscript{T} could not be assigned to any of the recognized genera. Consequently, strain P2\textsuperscript{T} should be considered to represent a novel species in a novel genus in the family Marinilabiliaceae.

Production of flexirubin-type pigments was assessed by the addition of 2 M KOH, according to Barbeyron et al. (2008). Accordingly, the colour change from brownish to light yellow (Fig. S2) was observed upon the addition of KOH to the cell pellet, as occurs in the case of flexirubin as the dominant pigment (Reichenbach, 1992; Reichenbach et al., 1974). Carotenoids were extracted from cell pellets in the dark with acetone as described by Denger et al. (2002) and the absorption spectrum was measured using a SmartSpec Plus spectrophotometer (Bio-Rad). The absorption spectra of acetone extracts exhibited a single maximum at 469 nm scanning from 440 to 540 nm (Fig. S3), which is not indicative of a carotenoid(s) (Reichenbach et al., 1974). The pigments of strain P2\textsuperscript{T} were distinguished from related genera of the family Marinilabiliaceae (Table 1).

In this study, we tried to detect genes for photosynthesis in strain P2\textsuperscript{T} using a PCR method. The target genes were *pufL* and *pufM* genes, and *puhA* gene coding subunit H of the reaction centre. If these genes could be found in strain P2\textsuperscript{T}, it could suggest that strain P2\textsuperscript{T} was a variant photosynthetic bacterium. The presence of photosynthetic genes (*pufL*, *pufM* and *puhA*) were determined using PCR as described by Uchino et al. (2002). Analysis of respiratory quinones was carried out by the Identification Service and Dr Brian Tindall, DSMZ, Braunschweig, Germany. Cell biomass of strain P2\textsuperscript{T} for polar lipid analysis was obtained from cultures grown in YTBC medium at 35 °C for 48 h. The cellular polar lipids were extracted according to the procedures described by Yassin et al. (1993) and separated on silica gel 60 plates (Merck) by using TLC and identified according to their reactions with the phosphate spray reagent of Dittmer & Lester (1964). The presence of *pufL*, *pufM* and *puhA* genes was not confirmed in strain P2\textsuperscript{T}. It can be concluded that strain P2\textsuperscript{T} lacks photosynthetic activities. The respiratory quinone of strain P2\textsuperscript{T} was menaquinone 7 (100\% of total.

**Fig. 1.** Neighbour-joining tree showing the phylogenetic positions of strain P2\textsuperscript{T} and other members of the family Marinilabiliaceae, based on 16S rRNA gene sequences. Bootstrap values based on 1000 replications are shown as percentages at branch nodes. GenBank accession nos of 16S rRNA sequences are given in parentheses. Bar, 0.02 nt substitution (K\textsubscript{nucl}) units.
The polar lipid composition of strain P2T was found to be predominantly phosphatidylethanolamine and a much less abundant unknown polar lipid (see Fig. S4).

Cell material for genomic DNA extraction was obtained after cultivation at 35 °C for 48 h in YTBC broth. The genomic DNA was extracted and purified using the method described by Syn & Swarup (2000). The DNA G+C content was determined by using HPLC of the deoxyribonucleosides as described by Mesbah & Whitman (1989). The G+C content of the genomic DNA of strain P2T was determined as 44.2 ± 1.0 mol%. The DNA G+C content of strain P2T was almost the same as the type strain, Alkaliflexus imshenetskii, and some results from carbon tests indicated that strain P2T could be distinguished from the closest type strain, Mangroviflexus xiamenensis gen. nov., sp. nov. of the family Marinilabiliaceae (Table 1).

Fatty acids from whole cells grown on YTBC medium at 35 °C for 48 h were extracted, saponified and esterified. The fatty acid methyl esters were analysed by using GC according to the instructions of the MIDI system (Sasser, 1997). The dominant fatty acids of strain P2T were iso-C15:0 (33.5%), anteiso-C15:0 (18.9%), C16:0 (5.4%), C16:0 3-OH (7.7%) and iso-C17:0 3-OH (9.3%), accounting for 65.5% of the total fatty acids. Minor amounts of iso-C13:0 (1.2%), C14:0 (2.2%), iso-C17:0 (1.7%) and C18:0 (1.1%) were also found in strain P2T. These results differentiated strain P2T from members of three related genera of the family Marinilabiliaceae, which all contained C15:0 and C16:0 3-OH and iso-C17:0 3-OH (Zhilina et al., 2004). In particular, the fatty acid C15:0 content of Alkaliflexus imshenetskii Z-7010T is increased to 39.0%. Strain P2T and Mangroviflexus Cys1T differed significantly in the content of C18:1ω2 and 3-hydroxyl fatty acids. The fatty acid profile of strain P2T was in good agreement with iso-C15:0 and anteiso-C15:0 of Anaerophaga thermohalophila Fru22T, except that Anaerophaga thermohalophila Fru22T contains minor amounts of 3-OH-C15:0, iso-C16:0 and C18:1ω1 while it lacks iso-C13:0 and iso-C17:0. The cellular fatty acid profiles of strain P2T and those of the type strains of Alkaliflexus imshenetskii Z-7010T, Mangroviflexus Cys1T and Anaerophaga thermohalophila Fru22T are compared in Table S1.

The suggestion that strain P2T was distinct from the Alkaliflexus/Marinilabiliota/Anaerophaga isolates was also supported by comparison of phenotypic characteristics (Table 1). Fatty acid profiles are a physiological feature that distinguish the present isolates from all three relatives (Alkaliflexus imshenetskii Z-7010T, Mangroviflexus Cys1T and Anaerophaga thermohalophila Fru22T). Most notably, the major fatty acid of strain P2T was iso-C15:0 (33.5%), while C15:0 (39.0%) was the primary fatty acid of Alkaliflexus imshenetskii Z-7010T (Zhilina et al., 2004). Moreover, strain P2T could be distinguished from the closest type strain, Alkaliflexus imshenetskii Z-7010T, in oxygen tolerance, catalase activity and the range of pH values for growth. Furthermore, results of G+C content experiments and some results from carbon tests indicated that strain P2T and Mangroviflexus Cys1T were distinct. Finally, obvious differences in formation of spheres and spore-like structures, salt tolerance, carbon assimilation and the temperature range for growth between strain P2T and Anaerophaga thermohalophila Fru22T also suggested that they were distinct taxa. Strain P2T lacked carotenoids and contained flexirubin pigment, which was not consistent with the properties of the family Marinilabiliaceae. Differences in physiological, biochemical and chemotaxonomic characteristics between strain P2T and the type strains of related species are given in Tables 1 and S1. On the basis of the distant phylogenetic relationship with related taxa, the unique chemotaxonomic characteristics, physiological and biochemical traits described above, it is evident that strain P2T is considered to represent a novel species of a new genus in the family Marinilabiliaceae, for which the name Mangroviflexus xiamenensis gen. nov., sp. nov. is proposed.

The type strain of Mangroviflexus xiamenensis is P2T (=CGMCC 1.5167T=DSM 24214T). Results of the morphological and physiological characterization are given in Table 1 and summarized in the species description.

**Description of Mangroviflexus gen. nov.**

Mangroviflexus [Man.gro.vi.fle.xus. N.L. n. mangrovum, mangrove; L. masc. n. flexus, a bending, turn, curve; N.L. masc. n. Mangroviflexus, a bending (curved) bacterium from mangrove].

Gram-negative, oxidase and catalase-negative. Cells are long and rod-shaped. Obligately anaerobic, mesophilic and moderately halophilic bacteria with growth at pH 5.0–8.5. Metabolism is strictly fermentative. Cells lack carotenoids and contain flexirubin pigment. Non-photosynthetic. Do not form fruiting bodies. The dominant fatty acids are iso-C15:0, anteiso-C15:0 and iso-C17:0 3-OH. The DNA G+C content of the type strain of the type species is about 44.0 ± 1.0 mol%. The genus is assigned phylogenetically to the family Marinilabiliaceae in the order Bacteroidales. The only known strain was isolated from offshore mangrove sediment. The type species is Mangroviflexus xiamenensis.

**Description of Mangroviflexus xiamenensis sp. nov.**

Mangroviflexus xiamenensis (xi.a.men.en’is. N.L. masc. adj. xiamenensis of or belonging to Xiamen).

Displays the following properties in addition to those given for the genus. Cells are slender rod-shaped (6.0–6.5 μm long and 0.25 μm wide). Oxidase-negative and strictly anaerobic. Gram-negative, non-spore-forming. Cells are motile. A yellow pigment similar to flexirubin is present. Carotenoids not detected. Dependence of colour on the presence of light during growth has not been observed. Grows at temperatures from 22 to 39 °C (optimum 35 °C) and at pH values between 5.0 and 8.5 (optimum pH 7.0–7.5). Grows in the presence of 0.2–3.5% (w/v) NaCl (optimum at 0.5–1.0%). The main fermentation products are propionate, acetate, hydrogen sulfide and succinate.
Utilizes cellobiose, glucose, xylose, arabinose, maltose, mannose and starch as sole carbon/nitrogen sources; does not utilize mannitol, trehalose, sorbitol, fructose, sucrose and glycerol. Utilization of lactose, rhamnose and galactose is variable. Used H₂/CO₂ with acetate, DL-lactate, pyruvate, yeast extract, Casamino acids and cysteine as electron donors but not succinate, L-malate, citrate, oxalate, formate, propionate, butyrate, methanol or ethanol. Served sulfate or sulfite as electron acceptors, but not sulfur, thiosulfate, oxygen, nitrite and nitrate. The closest phylogenetic relatives are Alkaliflexus inshenetskii, M. salmonicolor and Anaerophaga thermohalophila (based on 16S rRNA sequences). Cellular fatty acids are dominated by iso-C₁₅:₀ (33.5 %), anteiso-C₁₅:₀ (18.9 %) and C₁₇:₀ 3-OH (13.3 %). The respiratory quinone is menaquinone-7 (100 % of total quinone) and the major polar lipid is phosphatidylethanolamine.

The type strain was isolated as a component of an anaerobic cellulose-degrading enrichement culture originating from Dongzhai Port in Hainan Island, China. The type strain is P²⁺ (=CGMCC 1.5167T=DSM 24214T). The DNA G+C content is 44 mol%. The habitat is anoxic organic-rich offshore mangrove sediment.

Acknowledgements

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References


