Mangrovibacter yixingensis sp. nov., isolated from farmland soil

Hao Zhang, Su-hui Guo, Bin Sun, Jing Zhang, Ming-gen Cheng, Qiang Li, Qing Hong and Xing Huang

Key Laboratory of Microbiology of Agricultural Environment, Ministry of Agriculture, College of life sciences, Nanjing Agricultural University, Nanjing, 210095, PR China

The family Enterobacteriaceae is a complex group, and the members of this family are widely distributed and share some common characteristics: rod-shaped, Gram-staining-negative, facultatively anaerobic, do not form endospores, catalase-positive and oxidase-negative (Brenner, 1984). At present, there are more than 35 genera belonging to this family (Rameshkumar et al., 2010). Scholars and researchers show that when some isolates of the family Enterobacteriaceae are associated with plants, they will have enormous beneficial impact on the growth and development of plants (Lodewyckx et al., 2002; Taghavi et al., 2009). They improve plant growth via nitrogen fixation, suppression of plant pathogens and production of phytohormones, enzymes and growth regulators such as ethylene, 1-aminoacyclopropane 1-carboxylic acid (ACC), auxins and indole-3-acetic acid (IAA) (Gyaneshwar et al., 2001; Kämpfer et al., 2005; Taghavi et al., 2009). At the same time, some species of the genus Enterobacter are notorious plant pathogens or human opportunistic pathogens (Zhu et al., 2011). For example, Enterobacter cloacae is pathogenic to plants, affecting elm trees, mung bean sprouts, coconut, orchid, corn and onion bulbs (Nishijima et al., 2007). Due to the pathogenic effects on agriculture and livestock, some species of this family are of significant importance for the economy (Janda & Abbott, 2006).

The genus Mangrovibacter was first proposed and studied by Rameshkumar et al. (2010). At the time of writing, only one species, named Mangrovibacter plantisponsor, had been isolated from a mangrove-associated wild rice. In this report, the isolation and taxonomic characterization of strain TULL-A<sup>T</sup> belonging to the genus Mangrovibacter is described. On the basis of our results obtained from this study, it can be considered that isolate TULL-A<sup>T</sup> represents a novel species of the genus Mangrovibacter of the family Enterobacteriaceae.

The sample for isolation was collected from farmland soil (31° 21' 36" N 119° 49’ 12" E) in Yixing city, Jiangsu province, China, diluted 10-fold using sterilized water, and plated on LB (Difco) agar at 30 °C for 2 days; a single colony was picked out and further purified by plate-streaking two or three times. Finally, the pure culture of...
strain TULL-A\(^T\) was preserved at \(-80\,\text{\degree C}\) in LB broth (Difco) supplemented with 15\% \((v/v)\) glycerol.

Strain TULL-A\(^T\) was cultivated on LB agar or in LB broth at 30 \(^\circ\text{C}\) for morphological, biochemical and physiological analysis. Gram staining was performed using the method described by Beveridge \textit{et al.} (2007). Cell morphology was observed using a transmission electron microscope (H-7650; Hitachi) after 4 h of growth on LB agar. Oxidase (tetramethyl-p-phenylenediamine dihydrochloride test) and catalase (\(\text{H}_2\text{O}_2\) test) activities were tested according to the methods of Dong & Cai (2001). Hydrolysis of gelatin, casein, starch, Tween 80 and DNA were carried out according to described methods (Reichenbach, 1992; Smibert & Krieg, 1994). Acid production from various carbohydrates, production of \(\text{H}_2\text{~S}\), nitrate reduction, glucose oxidation/fermentation, indole production and hydrolysis of aesculin and urea were performed using API 50CH (medium E), API 20NE and API 20E kits (bioMérieux). Denitrification and methyl red tests were performed using standard methods as described by Smibert & Krieg (1994). Sensitivity and resistance to antibiotics were evaluated on LB agar using antibiotic discs (Hangzhou Microbial Reagent). Strains were incubated in an anaerobic triangular flask for anaerobic growth analysis. To determine the optimal temperature, pH and concentration of \(\text{NaCl}\) for growth, cultures were grown at 5, 15, 20, 25, 30, 37, 42 and 45 \(^\circ\text{C}\), 0–11\% \((w/v)\) \(\text{NaCl}\) (1\% intervals) and pH 3–10 (1 pH unit intervals). Additional properties were determined using API 20NE, API ZYM and API ID 32GN kits (bioMérieux).

Unless otherwise mentioned, strain TULL-A\(^T\) and the type strain \(M.\) \textit{plantisponsor} MSSRF40\(^T\) were cultivated in LB broth at 30 \(^\circ\text{C}\) until the growth reached exponential phase for the analysis of fatty acids, respiratory quinones, polar lipids, DNA G + C content and DNA–DNA hybridization. Cells were collected by centrifugation, washed three times with sterilized water and freeze-dried. Fatty acids were extracted and analysed according to the instructions of the Sherlock Microbial Identification System (MIDI). An Agilent Technologies 7890A GC System configured with an Agilent gas phase capillary column (0.2 mm \times 25 m, ultra 25 \% Phenyl Methyl Silox) was used. After saponification, methylation, extraction and purification, fatty acid methyl ester mixtures were separated by the Sherlock Microbial Identification System and the results were analysed by using MIDI Sherlock version 6.1. The respiratory quinones of the strains were studied according to the method of Collins \textit{et al.} (1977), including collection of bacteria, extraction and purification of quinones, and separated by HPLC. Analysis of the polar lipids by two-dimensional TLC was carried out by the identification service of the DSMZ (Braunschweig, Germany). The DNA G + C content was determined by using reversed-phase HPLC using methyl alcohol/isopropyl alcohol \((2 : 1, v/v)\) as the solvent and a flow rate of 1.0 ml min\(^{-1}\) according to the protocol of Mesbah \textit{et al.} (1989). DNA–DNA hybridization was performed according to De Ley \textit{et al.} (1970), through DNA extraction, purity analysis and calculation of the degree of hybridization based on the renaturation rate.

Cells of strain TULL-A\(^T\) were Gram-staining-negative rods (approx. 0.8–1.0 \(\mu\text{m}\) in diameter and 1.8–3.0 \(\mu\text{m}\) in length when cultured on LB agar at 30 \(^\circ\text{C}\) for 4 h) (Fig S1, available in the online Supplementary Material) with peritrichous flagella, facultatively anaerobic, oxidase-negative, catalase-positive, negative for the methyl red test, arginine dihydrolase activity, ornithine decarboxylase and aesculin hydrolysis, and positive for the Voges–Proskauer reaction. Colonies on LB were circular, smooth, creamy white and 1–2 mm in diameter within 1 day at 30 \(^\circ\text{C}\); swarming was not detected. Growth occurred at 15–42 \(^\circ\text{C}\), pH 4.0–9.0 and in the presence of 0–6\% \(\text{NaCl}\), optimally at 30 \(^\circ\text{C}\), pH 7.0–8.0 and 0\% \(\text{NaCl}\). Strain TULL-A\(^T\) was positive for nitrate reduction, citrate utilization and hydrolysis of Tween 80, but negative for urease, lysine decarboxylase, tryptophan deaminase, indole production, hydrogen sulphide production, denitrification, and hydrolysis of DNA, starch, casein and gelatin. Strain TULL-A\(^T\) produced acid from sucrose, raffinose, amygdalin, sorbitol, glycerol, ribose, \(\text{D-xylose}\), galactose, glucose, fructose, mannose, rhamnose, mannitol, methyl \(\text{D-\text{glucoside}}\), \(\text{N-acytylglucosamine, arbutin, cellobiose, malto, melibiose, trehalose, gentiobiose, D-arabitol, L-arabinose, salicin and 5-ketogluconate. Delayed acid production (48 h) in API 50 CH test strips was observed for lactose, turanose, gluconate and 2-ketogluconate. Strain TULL-A\(^T\) was negative for acid production from erythritol, \(\text{D-arabinose, adonitol, L-xylose, methyl }\text{D-xylose}, \text{sorbose, dulcitol, inositol, methyl }\text{D-mannoside, inulin, melezitose, starch, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose and L-arabitol. Cells were resistant to oxacillin, vancomycin, medemycin, erythromycin and clindamycin, but sensitive to cefoperazone, penicillin \(\text{G, ofloxacin, cefuroxime, ceftazidime, polymyxin B, ciprofloxacin, ceftriaxone, gentamicin, kanamycin, amikacin, cefazolin, furadantin, streptomycin, aztreonam, neben, cefafolin, ampicillin, tetracycline, chloramphenicol, norfloxacin, pipercillin, cefotaxin, spectinomycin, minocycline, cefepime, levofloxacin, cefotaxime and chemitrim. In the API ZYM strip, alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and }\text{β-galactosidase activities were present, but esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, \(\text{α-chymotrypsin, }\text{α-galactosidase, }\text{α-glucosidase, }\text{β-glucosidase, }\text{β-glucuronidase, }\text{N-acytyl-β-glucosaminidase, }\text{α-mannosidase and }\text{β-fucosidase activities were absent. The results of API ID 32GN tests and different phenotypic characteristics between strain TULL-A\(^T\) and }\text{M. plantisponsor} \text{MSSRF40}\(^T\) \text{are respectively listed in the species description and Table 1. Differences in fatty acids between strain TULL-A\(^T\) and }\text{M. plantisponsor} \text{MSSRF40}\(^T\) \text{are shown in Table 2. The major fatty acids (>5\%) of strain TULL-A\(^T\) were }\text{C16 : 0, summed feature 3 (comprising C16 : 1ω7c and/or C16 : 1ω6c), summed feature 8 (comprising C18 : 1ω7c/C18 : 1ω6c),...}
**Table 1. Different characteristics of strain TULL-AT$^T$ and M. plantisponsor MSSRF40$^T$**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Produces acid from (API 50 CH):</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>5-Ketogluconate</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Salcin</td>
<td>+</td>
<td>+ (D)*</td>
</tr>
<tr>
<td>Lactose</td>
<td>+ (D)*</td>
<td>−</td>
</tr>
<tr>
<td>Turanose</td>
<td>+ (D)*</td>
<td>−</td>
</tr>
<tr>
<td>Enzyme activity (API ZYM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetyl-β-glucosaminidase</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of (API ID 32GN):</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sodium malonate</td>
<td>+</td>
<td>+ (D)</td>
</tr>
<tr>
<td>Salcin</td>
<td>+</td>
<td>+ (D)*</td>
</tr>
<tr>
<td>Trisodium citrate</td>
<td>+</td>
<td>+ (D)*</td>
</tr>
<tr>
<td>L-Proline</td>
<td>+</td>
<td>+ (D)*</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>−</td>
<td>+ (D)*</td>
</tr>
<tr>
<td>2-Ketogluconate</td>
<td>+</td>
<td>+ (D)*</td>
</tr>
<tr>
<td>Voges–Proskauer reaction (API 20E)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine dehydrodase activity (API 20NE)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 42 °C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 7 % NaCl</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Hydrolysis of Tween 80</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>52.5</td>
<td>50.1</td>
</tr>
<tr>
<td>Nitrogen fixing capacity (nmol ethylene ml⁻¹ h⁻¹)</td>
<td>15.57±1.12</td>
<td>32.68±1.42</td>
</tr>
</tbody>
</table>

C₁₄:0 summed feature 2 (comprising C₁₄:0 3-OH/iso-C₁₆:1 I) and summed feature 4 (comprising iso-C₁₇:1 I and/or anteiso-C₁₇:1 B). The major respiratory quinone of strain TULL-AT$^T$ and M. plantisponsor MSSRF40$^T$ were Q8. Both strain TULL-AT$^T$ and MSSRF40$^T$ contained phosphatidyethanolamine and phosphatidylglycerol as the predominant polar lipids. Both of the strain TULL-AT and MSSRF40T contained phosphatidyethanolamine, phosphatidylglycerol as the predominant polar lipid. Aminophospholipid and two unidentified phospholipids (PL3, PL4) were present in the strain TULL-AT, which were not detected in the strain MSSRF40T. Diphasphatidylglycerol was only found in the strain MSSRF40T (Supplementary Fig. S4). The DNA G+C content of strain TULL-AT$^T$ was 52 mol%. The DNA–DNA relatedness between strain TULL-AT$^T$ and M. plantisponsor MSSRF40$^T$ was 35.10±1.41 %, which is lower than the value of 70 % that is commonly accepted for definition of a novel bacterial species (Stackebrandt & Goebel, 1994).

Genomic DNA was extracted according to the method of Ausubel et al. (1995). The 16S rRNA gene was amplified by the PCR primer pair 27f/1492r (Lane, 1991; Marchesi et al., 1998) and compared with available sequences using the EzTaxon-e server (Kim et al., 2012). The rpoB, gyrB and hsp60 genes were amplified as described by Mollet et al. (1997), Dauga (2002) and Iversen et al. (2004). For all of the above-mentioned genes, PCR amplification was carried out in a total volume of 50 μl containing 1 μl template DNA (50–100 ng), 0.5 μl Taq DNA polymerase, 5 μl 10 × PCR buffer, 3 μl dNTP mixture (2.5 mM) and 1 μl each of the forward and reverse primers (25 μM) and brought to a final volume of 50 μl using double-sterilized distilled water. The gene sequences of rpoB, gyrB and hsp60 were inferred using the neighbour-joining method (Saitou & Nei, 1987), and phylogenetic trees of the 16S rRNA gene sequences were reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods and bootstrap analysis was based on 1000 resamplings. The MEGA5.0 package (Tamura et al., 2011) was used for all analyses.

An almost-complete 16S rRNA gene sequence (1464 bp) of strain TULL-AT$^T$ was amplified by the PCR primer pair described above. Phylogenetic trees were reconstructed using this sequence and those of other members of the family Enterobacteriaceae and Plesiomonas shigelloides ATCC 14029$^T$ was chosen as an outgroup. Sequences comparison based on 16S rRNA gene showed that strain TULL-AT$^T$ had the highest similarity to M. plantisponsor MSSRF40$^T$ (99.6 %), followed by Salmonella enterica subsp. diarizonae DSM 14847$^T$ (96.8 %), Cronobacter condimenti 1330$^T$.

**Table 2. The differences of fatty acids between strain TULL-AT$^T$ and M. plantisponsor MSSRF40$^T$**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C₁₆:0</td>
<td>0.32</td>
<td>ND</td>
</tr>
<tr>
<td>C₁₇:0 cyclo</td>
<td>0.88</td>
<td>1.87</td>
</tr>
<tr>
<td>C₁₈:0</td>
<td>0.58</td>
<td>1.44</td>
</tr>
<tr>
<td>C₁₈:0 9c</td>
<td>2.14</td>
<td>3.87</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>22.99</td>
<td>18.65</td>
</tr>
<tr>
<td>Summed feature 4*</td>
<td>5.58</td>
<td>6.99</td>
</tr>
</tbody>
</table>

*Summed feature 3 comprises C₁₆:1 9c and/or C₁₆:1 9c. Summed feature 4 comprises iso-C₁₇:1 I and/or anteiso-C₁₇:1 B.
(96.8 %), Salmonella enterica subsp. salamae DSM 9220$^T$ (96.8 %) and Cronobacter muytjensi ATCC 51329$^T$ (96.5 %). Strain TULL-A$^T$ clearly formed a branch with M. plantisponsor MSSRF40$^T$ in the neighbour-joining phylogenetic tree (Fig. 1). Similar results were also found in the maximum-parsimony and maximum-likelihood phylogenetic tree (Fig. 1). In addition, strain TULL-A$^T$ had the highest $rpoB$, $gyrB$ and $hsp60$ gene sequence similarity with strain M. plantisponsor MSSRF40$^T$ (95.5, 94.1 and 93.4). According to the neighbour-joining phylogenetic trees, sequences of the three genes of TULL-AT all formed a clade with the respective sequences of M. plantisponsor MSSRF40$^T$ (Figs S5, S6 and S7).

The acetylene reduction assay was used to test for nitrogen fixation of strain TULL-AT and MSSRF40$^T$ (15.57 ± 1.12 nmol ethylene ml$^{-1}$ h$^{-1}$), which indicated that strain TULL-A$^T$ was able to fix atmospheric nitrogen but showed weaker capacity than strain MSSRF40$^T$ (15.57 ± 1.12 nmol ethylene ml$^{-1}$ h$^{-1}$). The gene $nifH$ was amplified using universal primers, nifH-1 GGTGTCGATCC(AGC)AGGGCCTGA(CT)TC(AGC)ACCCG, nifH-2 CTG(AGC)GGCTTGTG(TC)TCGCGGAT(GC)GGCATGGC and showed the highest similarity to dinitrogenase reductase Fe protein (Bacillus cereus, 98 %), dinitrogenase reductase Fe protein (Poenibacillus sp. g2, 98 %) and dinitrogenase reductase Fe protein (Bacillus megaterium, 98 %), followed by NiFH, partial (Klebsiella sp. BM92, 97 %) and dinitrogenase reductase Fe protein (Bacillus sp. w5, 97 %).

Phylogenetic analysis and chemotaxonomic characteristics (major fatty acids, predominant respiratory quinone, DNA G + C content) unequivocally supported the placement of strain TULL-A$^T$ within the genus Mangrovibacter. However, differences in phenotypic characteristics between strain TULL-A$^T$ and M. plantisponsor MSSRF40$^T$ suggested that strain TULL-A$^T$ could be differentiated from

![Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain TULL-A$^T$ to closely related strains. The 16S rRNA gene sequence of Plesiomonas shigelloides ATCC 14029$^T$ was used as an outgroup. Bootstrap values (based on 1000 replications, only values above 50 %) are shown at branch points. Bar, 0.005 substitutions per nucleotide position.]
recognized species of the genus *Mangrovibacter*. Therefore, morphological, physiological and chemotaxonomic characteristics together with DNA–DNA hybridization results support the suggestion that strain TULL-Å\(^1\) represents a novel species of the genus *Mangrovibacter*, for which the name *Mangrovibacter yixingensis* sp. nov. is proposed.

**Description of Mangrovibacter yixingensis sp. nov.**

*Mangrovibacter yixingensis* (yi.xing.en’sis. N.L. masc. adj. *yixingensis* referring to Yixing in the Jiangsu province, China, the area from where the type strain was isolated). Cells are Gram-staining-negative, facultatively anaerobic rods (approx. 0.8–1.0 \(\mu\)m in diameter and 1.8–3.0 \(\mu\)m in length when cultured on LB agar at 30 °C for 4 h) with peritrichous flagella. The methyl red test, oxidase activity, ornithine decarboxylase and aesculin hydrolysis are negative, catalase activity, the Voges–Proskauer reaction and nitrogen fixation are positive. Colonies on LB are circular, smooth, creamy white and 1–2 mm in diameter within 18 h at 30 °C; swarming is not detected. Growth occurs at 15–42 °C (optimally at 30 °C), in the presence of 0–6% NaCl (0% optimally, no growth at 7% NaCl) and at pH 4.0–9.0 (optimally at pH 7.0–8.0). Nitrate reduction and citrate utilisation and hydrolysis of Tween 80 are positive, and urease, lysine decarboxylase, tryptophan deamination, and hydrolysis of DNA, starch, casein and gelatin are negative. Produces acid from sucrose, raffinose, amygdalin, sorbitol, glycerol, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, mannitol, methyl \(\alpha\)-D-glucoside, N-acetylgalactosamine, arbutin, cellobiose, maltose, melibiose, trehalose, gentiobiose, D-arabitol, L-arabinose, salicin and 5-ketogluconate. Delayed acid production (48 h) in API 50 CH test strips for lactose, turanose, gluconate and 2-ketogluconate. Negative for acid production from erythritol, D-arabinose, adonitol, L-xylose, methyl \(\beta\)-D-xyloside, sorbose, dulcitol, inositol, methyl \(\alpha\)-D-mannoside, inulin, melezitose, starch, glycogen, xylitol, D-xylose, D-tagatose, D-fucose, L-fucose and L-arabinose (API 50 CH). Can utilize L-rhamnose, N-acetylgalactosamine, D-ribose, sucrose, maltose, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, L-serine, D-mannitol, D-glucose, salicin, melibiose, D-sorbitol, D-arabinose, potassium 2-ketogluconate, L-proline, D-mannose, potassium gluconate and malic acid; weakly positive for utilization of sodium malonate and trisodium citrate, but not for inositol, itaconic acid, suberic acid, glycogen, 3-hydroxybenzoic acid, L-fucose, propionic acid, capric acid, valeric acid, histidine, 3-hydroxybutyric acid, 4-hydroxybenzoic acid, adipic acid or phenylacetic acid (API 20NE and API ID 32GN test strips). In the API ZYM strip, the activity of alkaline phosphatase, esterase (C4), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and \(\beta\)-galactosidase are present. But esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, \(\alpha\)-chymotrypsin, \(\alpha\)-galactosidase, \(\beta\)-glucuronidase, \(\alpha\)-glucosidase, \(\beta\)-glucosidase, N-acetyl-\(\beta\)-glucosaminidase, \(\alpha\)-mannosidase and \(\beta\)-fucosidase activity are absent. The major fatty acids are C16:0, summed feature 3 (comprising C16:1ω7c and/or C16:1ω6c), summed feature 8 (comprising C18:1ω7c/C18:1ω6c), C14:0, summed feature 2 (comprising C14:0 3-0H/iso-C16:1ω1) and summed feature 4 (comprising iso-C17:1ω and/or anteiso-C17:1ω). The major respiratory quinone is Q8. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and aminophos-aphospholipid.

The type strain, TULL-Å\(^1\) (=ACCC 19709\(^T\)=KCTC 42181\(^T\)), was isolated from farmland soil in Xing city, Jiangsu province, China. The DNA G+C content of the type strain is 52 mol%.

**Acknowledgements**

This work was supported by the National High Technology Research and Development Program of China (2012AA10403), the Project for Science and Technology of Jiangsu Province (BE2012749).

**References**


http://ijs.sgmjournals.org