Pleomorphomonas diazotrophica sp. nov., an endophytic N-fixing bacterium isolated from root tissue of Jatropha curcas L.

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A novel aerobic, non-motile, pleomorphic, Gram-negative and nitrogen-fixing bacterial strain, designated R5-392T, was isolated from surface-sterilized root tissue of Jatropha curcas. The organism grew optimally at 30 °C in media containing 1 % (w/v) NaCl and at pH 6.0–8.0. The predominant ubiquinone was Q-10 and the major fatty acids were C\textsubscript{18:1}v\textsubscript{7}C and C\textsubscript{16:0} cyclo C\textsubscript{8}C. The DNA G+C content was 63.2 mol%. Analysis of the 16S rRNA gene sequence suggested that strain R5-392T is affiliated with the order Rhizobiales within the class Alphaproteobacteria and is most closely related to Pleomorphomonas oryzae F-7\textsuperscript{T} (98.8 % similarity) and Pleomorphomonas koreensis Y9\textsuperscript{T} (98.3 % similarity). Analysis of partial nifH gene sequences also revealed a monophyletic lineage within the class Alphaproteobacteria, and strain R5-392T was most closely related to P. oryzae F-7\textsuperscript{T} (98 %). Highest nitrogenase activity was detected in the presence of low-level organic nitrogen or in the presence of nitrogenase co-factors (Fe/Mo) in N-free media. Phenotypic and chemotaxonomic data suggest that strain R5-392T represents a novel species within the genus Pleomorphomonas, for which the name Pleomorphomonas diazotrophica sp. nov. is proposed. The type strain is R5-392T (=KACC 16233\textsuperscript{T} =DSM 25022\textsuperscript{T}).

The genus Pleomorphomonas belongs to the order Rhizobiales, which includes plant and animal pathogens, plant endosymbionts, methane-oxidizing bacteria and, notably, a large number of nitrogen-fixing species. At the time of writing, there are two recognized species of the genus Pleomorphomonas, Pleomorphomonas oryzae (Xie & Yokota, 2005) from rice and Pleomorphomonas koreensis from a contaminated culture of a phototrophic bacterium (Im et al., 2006). Both are nitrogen-fixing species. All plants in nature harbour a diverse community of endophytic bacteria that may promote plant growth directly or indirectly by secreting plant-growth substances, supplying a nitrogen source through biological nitrogen fixation, solubilizing phosphorus, degrading growth inhibitors and suppressing pathogen attack (Rosenblueth & Martinez-Romero, 2006).

Jatropha (Jatropha curcas L.) is a small woody plant belonging to the family Euphorbiaceae that is endemic to tropical America. It has attracted a lot of attention in recent years due to its high potential as a crop for biofuel production (Divakara et al., 2010). During a study on the diversity and a search for growth-promoting bacteria in Jatropha, we found that Jatropha tissues were associated with several novel bacterial species (Madhaiyan et al., 2013a, b). Here, we characterize a nitrogen-fixing strain, designated R5-392\textsuperscript{T}, that exhibited the typical morphological characteristics of the genus Pleomorphomonas. Using a polyphasic taxonomic approach, we confirmed its position as a representative of a novel species within the genus Pleomorphomonas, for which the name Pleomorphomonas diazotrophica sp. nov. is proposed.

Strain R5-392\textsuperscript{T} was isolated from surface-sterilized root tissue of Jatropha curcas L. B1-2 collected from the Agrotechnology Experimental Station in Singapore in September 2009. The surface-sterilized roots were then macerated, diluted serially with 10 mM MgSO\textsubscript{4} and plated.
onto R2A medium (Difco Laboratories) and incubated at 30 °C. Colonies that appeared on R2A plates after 72 h were subcultured to obtain pure cultures. The isolate R5-392\textsuperscript{T} was routinely cultured on R2A plates at 30 °C under aerobic conditions and stored frozen at −80 °C in 15% (v/v) DMSO. Cell morphology was examined under scanning (JEOL-JSM-6360) and transmission electron microscopes (JEOL-JEM-1230). Cell wall structure and poly-β-hydroxybutyrate granules were imaged by transmission electron microscopy. All physiological characteristics including growth at different temperatures (4−45 °C), NaCl levels (1−5%) and pH (pH 4.0−10.0) were tested with cells cultured on R2A at 30 °C, unless otherwise stated. Gram staining and assays for motility and enzymic activities of catalase, oxidase, amylase, cellulase, xylanase, proteases and tyrosinase were determined as described by Kim et al. (2012b). Other physiological and biochemical characteristics, tested using the API ZYM and API 20NE galleries (bioMérieux), and nutritional features, determined by incubating Biolog GN2 microtitre plates (28 °C, 7 days), were carried out following the manufacturer’s instructions. Susceptibility to antibiotics was determined by spreading the bacterial suspension (OD\textsubscript{600} ~1.0) onto R2A plates supplemented with different concentrations (0, 25, 50, 100 and 250 μg ml\textsuperscript{-1}) of antibiotics and incubating for 3 days at 30 °C.

Genomic DNA extractions were carried out according to a standard protocol (Wilson, 1987). The 16S rRNA gene was amplified using universal primers 27F and 1492R (DeLong, 1992). Cycling conditions were as follows: 95 °C for 10 min, 30 cycles of 95 °C for 1.5 min, 55 °C for 1.5 min and 72 °C for 1.5 min, and final extension at 72 °C for 10 min. Sequencing was carried out with a Big Dye Terminator cycle sequencing kit (Perkin Elmer) using primers 27F (5'-AGAGTTTGATCCTGCGCTCAG-3'), 1492R (5'-GCGTACCTTGTTACGACTT-3'), 785F (5'-GGTATTAGATACCTGGTA-3'), 518R (5'-GATATTACGCGCTGCTG-3') and 1100R (5'-GGTGGTGGCTGTTTGTG-3') with an Applied Biosystems 3730 XL DNA sequencer (HITACHI). Sequence similarity was analysed against the EzTaxon-e Database (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012a) and aligned using the CLUSTAL W tool in MEGA version 5.05 (Tamura et al., 2011). Phylogenetic analyses were performed by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods using MEGA version 5.05 (Tamura et al., 2011) with bootstrap values set at 1000 replications (Felsenstein, 1985).

Nitrogen-fixing activity was determined by growing strains in 40 ml nitrogen-free medium (DSMZ medium no. 3) contained in a 125 ml serum bottle (Wheaton Industries) with purified acetylene (15%, v/v) incubated for up to 96 h at 30 °C as described by Im et al. (2006). Gas samples (0.5 ml) removed at regular intervals with a PTFE-syringe (Hewlett Packard) were analysed in a gas chromatograph (GC 6890N; Agilent Technologies) with an FID operated under the following conditions: carrier gas, He, 35 ml min\textsuperscript{-1}; detector temperature, 200 °C; column, GS-Alumina (30 m × 0.53 mm I.D.); pressure, 27.6 kPa. Ethylene produced by the bacteria was quantified by using a standard ethylene (C\textsubscript{2}H\textsubscript{4}; product number 00489; Sigma-Aldrich) curve prepared in duplicate in concentrations ranging from 1−1000 nmol. Protein concentration was determined with a modified Lowry method using BSA as standard. For nitrogenase switch-off/switch-on assay, R5-392\textsuperscript{T} cells were grown in N-free medium containing different levels (0−10 μM) of ammonium chloride and nitrogenase co-factors (Fe/Mo). PCR amplification of a partial nifH fragment was performed using the method of Pinto-Tomás et al. (2009). The cycling conditions were the following: 95 °C for 5 min, 40 cycles of 94 °C for 11 s, 92 °C for 15 s, 54 °C for 8 s, 56 °C for 30 s, 74 °C for 10 s and 72 °C for 10 s, and final extension at 72 °C for 10 min. PCR products were purified with a QIAquick gel extraction kit (Qiagen) and sequenced. The DNA sequences were compared against the NCBI database by BLAST.

Quinones and polar lipids were extracted and analysed according to Minnikin et al. (1984). For analysis of whole-cell fatty acid composition, strains were grown on R2A media for 2 days at 30 °C to reach stationary phase, except strain P. koreensis KCTC 12246\textsuperscript{T}, which was cultured for 4 days to reach stationary stage. Cell cultures of comparable optical density (OD\textsubscript{600}=0.8−1.0) were harvested for fatty acid analysis. The cellular fatty acids were extracted and analysed by GC (model 6890; Hewlett Packard) according to the protocol of the Sherlock Microbial Identification System (Sasser, 1990). The fatty acid methyl esters were identified and quantified by using the TSBA 6 database (version 6.10) of the Sherlock Microbial Identification System.

DNA G+C contents (mol%) were determined by HPLC analysis of deoxyribonucleosides as described by Msesbah et al. (1989), using a reversed-phase column (Supelcosil LC-18 S; Supelco). DNA–DNA hybridization tests were carried out using the filter hybridization method as described by Seldin & Dubnau (1985). Probe labelling was conducted by using the non-radioactive DIG-High prime system (Roche). Hybridized DNA was visualized using the DIG luminescent detection kit (Roche). DNA–DNA relatedness was quantified using a densitometer (Bio-Rad).

Cells of strain R5-392\textsuperscript{T} are aerobic, Gram-negative, pleomorphic, non-motile rods (1.4−2.6 μm long, 0.4−0.5 μm wide; Fig. 1) that are catalase- and oxidase-positive. Colonies are circular, smooth, convex, translucent and pale to white in colour on R2A agar plates, colourless on N-free agar medium and do not grow on KB, LB, 2 × YT or tryptic soy agar. The diameters of colonies on R2A and N-free agar plates were 0.3−0.6 and 0.4−0.7 mm, respectively, after 3 days at 30 °C. Strain R3-392\textsuperscript{T} was able to grow at 20−37 °C (optimum temperature, 30 °C) and pH 6.0−8.0 and exhibited growth in R2A media containing 0−1% (w/v) NaCl. There was no growth at temperatures >37 °C, pH ≤5.0 or ≥8.5, and with ≥2% (w/v) NaCl. Strain R5-392\textsuperscript{T} showed very similar phenotypic features and characteristics to reference strains, with some differences in respect to the

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production of urease, nitrate reduction and the utilization of some compounds (see species description and Table 1). The 16S rRNA gene sequence of strain R5-392T determined in this study was a continuous stretch of 1443 bp. A sequence similarity calculation performed after neighbour-joining analysis showed that strain R5-392T is most closely related to strains \(P.\ oryzae\) (98.8%) and \(P.\ koreensis\) (98.3%). Lower levels of similarity (<92%) were found with respect to all available sequences of species of the order \(Rhizobiales\). Phylogenetic analysis of the 16S rRNA gene sequences revealed that strain R5-392T formed a distinct lineage within the genus \(Pleomorphomonas\) (Figs 2 and S1). In order to determine if the new isolate constituted a novel species of the genus \(Pleomorphomonas\), we carried out DNA–DNA hybridization studies between reference strains of species of the genus \(Pleomorphomonas\) and strain R5-392T. Similarity values of 48% (reciprocal, 52%) and 45% (reciprocal, 52%) were obtained with \(P.\ oryzae\) KCTC 12245\(^T\) and \(P.\ koreensis\) KCTC 12246\(^T\), respectively. These values are below the threshold value (70%) suggested for species delineation.

![Fig. 1.](http://ijs.sgmjournals.org) (a) Pleomorphic rods (see long arrows, long rod shaped to bent and irregularly shaped cells) of strain R5-392\(^T\) by scanning electron microscopy. Bar, 1 \(\mu\)m. (b) and (c) Transmission electron micrographs of ultrathin section of strain R5-392\(^T\) showing cell wall structure and poly-\(\beta\)-hydroxybutyrate granules (see short arrows). Bars, 1 \(\mu\)m (b) and (c) 0.5 \(\mu\)m.

### Table 1. Differential characteristics of strain R5-392\(^T\) and closely related members of the genus \(Pleomorphomonas\)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Cell shape</td>
<td>Pleomorphic rods</td>
<td>Pleomorphic rods</td>
<td>Rods</td>
</tr>
<tr>
<td>pH range for growth ((^\circ\text{C}))</td>
<td>6–8</td>
<td>5–8</td>
<td>6–8</td>
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<tr>
<td>Optimum growth temperature ((^\circ\text{C}))</td>
<td>30</td>
<td>28–30</td>
<td>30</td>
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<tr>
<td>NaCl tolerance (%)</td>
<td>1</td>
<td>2</td>
<td>1</td>
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<td>Assimilation of:</td>
<td></td>
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<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
<td>w</td>
<td>–</td>
<td>w</td>
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<tr>
<td>(\alpha)-Mannosidase</td>
<td>w</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Nitrate reduction to nitrite ((\text{NO}_2^+))</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Glucose fermentation</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Activity of:</td>
<td></td>
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<tr>
<td>Urease (URE)*</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>(N)-Acetylglucosamine (NAG)</td>
<td>w</td>
<td>+</td>
<td>w</td>
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<td>Utilization of:</td>
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<tr>
<td>Potassium gluconate (GNT)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Malic acid (MLT)</td>
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<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>63.2</td>
<td>63.1</td>
<td>65.1</td>
</tr>
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</table>

*Discrepancies were found between this study and Im et al. (2006).
(Wayne et al., 1987), indicating that strain R5-392<sup>T</sup> represents a novel species of the genus *Pleomorphomonas*.

Members of the genus *Pleomorphomonas* were able to grow in N-free media and the presence of *nifH* gene sequences in these strains makes them considered diazotrophs (Xie & Yokota, 2005; Im et al., 2006). The N-fixation capability of the strain R5-392<sup>T</sup> was confirmed by a multidisciplinary approach. The *nifH* gene specific PCR yielded the expected 409 bp product from strain R5-392<sup>T</sup>. Sequencing of the PCR fragment showed that it encodes a polypeptide of 130 aa with 100% similarity to the ATP-dependent reductase or nitrogenase iron protein (nitrogenase component II, dinitrogen reductase, *nifH* protein) of *P. oryzae* F-7<sup>T</sup>, which has been used as a marker for nitrogenase (Zehr & Capone, 1996). Similarity searches in NCBI database
indicated that the nifH gene sequence of R5-392<sup>T</sup> showed the highest similarity to those of the nifH genes of <i>P. oryzae</i> strains F4 and F7<sup>T</sup> (93 % and 92 %, respectively) (Fig. S2). Strain R5-392<sup>T</sup> grown in N-free medium effectively reduced acetylene and exhibited maximum nitrogenase activity of >300 nmol C<sub>2</sub>H<sub>4</sub> ml<sup>−1</sup> in the headspace gas sample after 96 h of incubation at 30 °C, with the population increase of >6.0 log c.f.u. ml<sup>−1</sup> (Fig. 3a). In comparison, close relatives of R5-392<sup>T</sup> (KCTC 12245<sup>T</sup> and KCTC 12246<sup>T</sup>) produced <10 nmol C<sub>2</sub>H<sub>4</sub> per mg of protein (data not shown). Low concentrations of nitrogen (0.1 mM NH<sub>4</sub>Cl) in the growth medium stimulated nitrogenase activity while higher concentrations (>1 mM NH<sub>4</sub>Cl) switched off nitrogenase activity (Fig. 3b). Supplementing the medium with nitrogenase co-factors such as Na<sub>2</sub>MoO<sub>4</sub>/FeSO<sub>4</sub>.7H<sub>2</sub>O at various concentrations showed that Fe<sup>2+</sup> was essential for nitrogenase activity with an optimum of about 0.1 mM whereas Mo stimulated the nitrogenase activity over a wide concentration range (up to 10 mM) (Fig. 3c).

The predominant respiratory quinone was Q-10 (86 %), with smaller amounts of Q-8 (14 %), which is consistent with other species of the genus <i>Pleomorphomonas</i> (Xie & Yokota, 2005; Im <i>et al.</i>, 2006). The major cellular fatty acids of strain R5-392<sup>T</sup> grown on R2A agar plates were summed feature 8 (C<sub>18:1ω7c/C18:1ω6c</sub>) (62.6 %), C<sub>16:0</sub> (11.7 %), C<sub>19:0</sub> cyclo ω8c (8.2 %), summed feature 2 (C<sub>14:0</sub> 3-OH/iso-C<sub>16:1ω</sub> I) (5.6 %), C<sub>18:0</sub> 3-OH (4.0 %) and C<sub>18:0</sub> (3.9 %). Strain R5-392<sup>T</sup> has summed feature 8 (C<sub>18:1ω7c/C18:1ω6c</sub>, C<sub>16:0</sub> and cyclo C<sub>19:0</sub>ω8c as the main cellular fatty acids, similar to other species of the genus <i>Pleomorphomonas</i> also have summed feature 8 (C<sub>18:1ω7c/C18:1ω6c</sub>, C<sub>16:0</sub> and C<sub>19:0</sub> cyclo ω8c as the major cellular fatty acids (Xie & Yokota, 2005; Im <i>et al.</i>, 2006). The fatty acid profile of strain R5-392<sup>T</sup> can be clearly differentiated from that of the closely related type strains of the genus <i>Pleomorphomonas</i>, supporting the proposal that strain R5-392<sup>T</sup> is a novel species (Table S1). Polar lipids of strain R5-392<sup>T</sup> consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, phosphatidylinomethylthanolamine, phosphatidylethanolamine and two aminophospholipids (AL1 and AL2) (Fig. S3). Reference strain KACC 11479<sup>T</sup> also showed the same pattern of polar lipids. Both strains are negative for Dragendorff reagent for choline-containing lipids (Fig. S3). The DNA G+C content of strain R5-392<sup>T</sup> was 63.2 mol%, which is within the range (62.1–65.1 mol%) for other species of the genus <i>Pleomorphomonas</i> (Table 1).

The genotypic and phenotypic data showed that strain R5-392<sup>T</sup> is a member of the genus <i>Pleomorphomonas</i>. However, it can be discriminated from closely related members of the genus <i>Pleomorphomonas</i> by its 16S rRNA gene sequence (98.8 % similarity), the presence of differentiating cellular fatty acids, carbon source oxidation profiles and enzyme activities, and low DNA–DNA hybridization value (45–48%). These data suggest that strain R5-392<sup>T</sup> represents a novel species of the genus <i>Pleomorphomonas</i>, for which the name <i>Pleomorphomonas diazotrophica</i> sp. nov. is proposed.

**Description of Pleomorphomonas diazotrophica sp. nov.**

<i>Pleomorphomonas diazotrophica</i> [di.ə.zo.tro’phi.ca. L. inseparable particle dis twice, doubly; N.L. n. azotum
nitrogen; N.L. pref. diazo- pertaining to dinitrogen; N.L. fem. adj. trophica (from Gr. fem. adj. tropikhê) nursing, tending; N.L. fem. adj. diazotrophica growing on dinitrogen, isolated from root tissue of *Jatropha curcas* L. [42x620] *J. curcas* L.

Cells are Gram-negative, non-motile, pleomorphic, ranging from long rod-shaped to bent and irregularly shaped cells, 1.4–2.6 × 0.4–0.5 μm in size (Fig. 2a, b). Poly-β-hydroxybutyrate granules are accumulated (Fig. 2c). Growth occurs on R2A agar, nutrient agar and M869 agar (weak) at 30 °C and pH 6.0–8.0 but not in KB, LB, 2YT or tryptic soy agar. Catalase and oxidase are positive. Growth occurs in the presence of 1% (w/v) NaCl, but not in the presence of ≥2% (w/v) NaCl. Growth occurs between 20 and 37 °C with an optimum at 30 °C. The pH range for growth is pH 6–8, with optimal growth at pH 7. Starch, casein, and aesculin hydrolysis are positive but gelatin, tyrosine, CM-cellulose and xylan hydrolysis are absent. Indole production, urease and β-galactosidase are positive. Nitrate reduction is negative. Solute carbon and energy sources tested are given in Table S2. API 20NE analysis and API ZYM test strip results are given in Table 1. The type strain is highly resistant (up to 250 μg ml⁻¹) to carbenicillin and ampicillin but sensitive (25 μg ml⁻¹) to kanamycin, tetracycline, hygromycin, spectinomycin and rifampicin. Moderate sensitivity to nalidixic acid (50 μg ml⁻¹) and chloramphenicol (100 μg ml⁻¹). The *nifH* gene for nitrogenase activity is present. The predominant ubiquinone is Q-10. The major fatty acids are summed feature 8 (C_{18:0}ω7c/C_{18:0}ω6c), C_{16:0} and C_{19:0} cyclo ω8c. The polar lipids comprise diphasphatidylglycerol, phosphatidylglycerol, phosphatidylinoline, phosphatidylmonomethylethanolamine, phosphatidylethanolamine and two aminophospholipids (AL1 and AL2).

The type strain, R5-392T (=KACC 16233T =DSM 25022T), was isolated from root tissue of *Jatropha curcas*, the sample being collected at the flowering stage from Jatropha farm, Agrotechnology Experimental Station, at Lim Chu Kang, Singapore. The DNA G+C content of the type strain is 63.2 mol%.

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**References**


