Rhizobium lemnæ sp. nov., a bacterial endophyte of Lemna aequinoctialis

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Bacterial strain L6-16T was isolated from Lemna aequinoctialis. Cells were Gram-stain-negative, rod-shaped and motile with monopolar flagella. The phylogenetic analysis of its nearly complete 16S rRNA gene sequence revealed that strain L6-16T was a member of the genus Rhizobium. Its closest relative was Rhizobium tainense PL-41T with a 16S rRNA gene sequence similarity value of 98.3 %. Sequence similarity analysis of the housekeeping recA and atpD genes showed low levels of sequence similarity (<93.9 %) between strain L6-16T and other species of the genus Rhizobium. Strain L6-16T was able to grow between pH 5 and 11 (optimum 7.0) and at temperatures ranging from 20 to 41 °C (optimum 30 °C). It tolerated NaCl up to 1 % (w/v) (optimum 0.5 %). C₁₈:₁ω7c and/or C₁₈:₁ω6c (summed feature 8; 79.5 %) were found as predominant cellular fatty acids. The DNA G+C content of strain L6-16T was 58.1 mol% (Tm). Based on low levels of DNA–DNA relatedness, strain L6-16T was distinct from members of phylogenetically related species including R. tainense PL-41T (38.3 ± 0.8 %), Rhizobium rosettiformans W3T (6.9 ± 0.4 %) and Rhizobium pseudorzea J3-A127T (12.3 ± 0.6 %). Strain L6-16T was unable to nodulate the roots of Phaseolus vulgaris, and nodC and nfh genes were not detected. The results obtained from phylogenetic analyses, phenotypic characterization and DNA–DNA hybridization indicated that strain L6-16T represents a novel species of the genus Rhizobium, for which the name Rhizobium lemnæ sp. nov. is proposed. The type strain is L6-16T (=NBRC 109339T =BCC 55143T).

The genus Rhizobium belongs to the family Rhizobiaceae and was originally described by Frank (1889) as a collection of stem and/or root nodulating bacteria. Previously, the source of isolation of species of the genus Rhizobium was generally root nodules of various leguminous plants. This led to the use of the nodulation activity and the specificity towards various plant hosts as natural classification at the species level. However, this means of classification has been proven unreliable because a number of non-symbiotic strains have been reported in various species of the genus Rhizobium (Young et al., 2001). Additionally, several strains have been isolated from the rhizosphere and internal tissues of non-leguminous plants including rice, wheat, maize and duckweed (Kittiwongwattana & Thawai, 2013; Peng et al., 2008; Rosenblueth & Martinez-Romero, 2004; Schloter et al., 1997; Zhang et al., 2011b). At the time of writing, more than 50 bacterial species have been characterized as members of the genus Rhizobium (Turdahon et al., 2013). The main interest in the members of the genus Rhizobium has been their plant growth promotion through the nitrogen-fixing activity (Yanni et al., 1997). Some species of the genus Rhizobium were also proposed to promote growth of non-leguminous plants through the production of the plant hormone indole-3-acetic acid and the ethylene-degrading enzyme ACC-deaminase (Bhattacharjee et al., 2012; Datta & Basu, 2000). However, previous studies have shown that some species of the genus Rhizobium are able to cause crown gall disease in plants as well as nosocomial infection in human (Kaselitz et al., 2012; Pulawska et al., 2012a, 2012b).

Members of the duckweed family are recognized as the smallest flowering plants. Currently, 38 different species have been classified into five different genera including Spirodea, Landoltia, Lemna, Wolffia and Wolfiella (Wang et al., 2010). However, the diversity of bacterial endophytes colonizing the internal tissues of these plant species is not well studied. In the present study, we report on the taxonomic characterization and classification of strain L6-16T that was isolated from Lemna aequinoctialis.
Healthy-looking *Lemna aequinoctialis* plants were obtained from a pond in Bangmueang district, Samutprakan Province, Thailand. After surface-sterilization with 10% sodium hypochlorite and a few drops of Tween 20, plants were rinsed with sterilized distilled water five times. Surface-sterilized plants were ground in sterilized distilled water, and the bacterial suspension was plated on 1/10 strength tryptic soy agar (TSA; LabM) and incubated at 30 °C for 7 days. A bacterial isolate designated strain L6-16T was isolated and purified by cross streaking on nutrient agar (NA; LabM) medium.

Genomic DNA of strain L6-16T was prepared as described by Araújo *et al.* (2002) and used for amplification of a nearly complete 16S rRNA gene fragment. The universal primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5’-TACGTTACCTTGTACGACTT-3’) (Lane, 1991) were used in the PCR, and the temperature profile was as follows: initial denaturation at 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 90 s; and final extension at 72 °C for 5 min. The sequence of the PCR product was obtained using the 27F, 1492R, 350F (5’-TACGGAGGCCGAGCAG-3’), 350R (5’-TACGGAGGCCGAGCAG-3’), 780F (5’-TACGGAGGCCGAGCAG-3’), 1100F (5’-GCAACGGAGCGGAAACCC-3’), 350R (5’-CGTACTTCCGCTAGTAC-3’) and 780R (5’-CTACGGAGTATTTAATC-3’) primers (Lane, 1991). Pairwise alignment of the nearly complete 16S rRNA gene sequence of strain L6-16T was performed using the EzTaxon server (Kim *et al.*, 2012). Partial sequences of housekeeping genes recA and atpD of strain L6-16T were also determined using primers and methods described previously by Islam *et al.* (2008) and Martens *et al.* (2008).

Partial sequences of the 16S rRNA, recA and atpD genes of recognized species of the genus *Rhizobium* were obtained from the GenBank database for multiple alignment analyses using the CLUSTAL W program, version 1.81 (Thompson *et al.*, 1994). The alignment was manually corrected to eliminate gaps and ambiguous nucleotides. Phylogenetic analysis was conducted using the neighbour-joining method (Saitou & Nei, 1987) in the programme MEGA version 5.2 (Tamura *et al.*, 2011), and evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980). Additional phylogenetic analyses using the maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods were also performed using the same program. Confidence levels for the clusters were determined using bootstrap analysis (Felsenstein, 1985) with 1000 resamplings.

Morphological characteristics of strain L6-16T were determined after growth on yeast mannitol agar (YMA) medium (1 g yeast extract, 10 g mannitol, 0.5 g K2HPO4, 0.1 g MgSO4, 7H2O, 0.05 g NaCl, 0.05 g CaCl2·2H2O, 0.006 g FeCl3·6 H2O, 20 g agar; pH 7.0) at 30 °C for 2 days. Additional cell morphology was observed under a transmission electron microscope (JEM-1230; JEOL). Phenotypic characteristics including utilization of different carbon sources, growth at different pH and temperature, and NaCl tolerance were examined and compared with those of reference strains. Carbon utilization tests were performed according to the methods described by Gao *et al.* (1994). The pH range (pH 4–12) for growth and NaCl tolerance (0.5–6%, w/v) were determined on yeast mannitol (YM) medium (YMA without agar) at 30 °C. Growth at various temperatures (20–45 °C) was examined on YMA medium. Resistance to penicillin G (10 U), streptomycin (10 μg) and gentamicin (10 μg) was tested on YMA medium using discs containing the antibiotics. The results of each test were determined after 48 h of incubation. Catalase, urease, gelatinase and cytochrome oxidase activities were determined according to methods described previously (Graham & Parker, 1964; Lindström & Lehtomäki, 1988; MacFaddin, 2000; Skerman, 1967). Cells of strain L6-16T and the reference strains were grown on TSA plates at 30 °C for 48 h and used for cellular fatty acid profile analysis. The analysis was performed using GLC according to the instructions of the Microbial Identification System (MIDI), Sherlock version 6.0 (Kämpfer & Kroppenstedt, 1996; Sasser, 1990). Cellular fatty acids were identified using the TSBA6 database of the MIDI system (Sasser, 1990).

Genomic DNA of strain L6-16T and the reference strains used for DNA G+C content analysis and DNA–DNA hybridization was prepared according to the method described by Marmur (1961). The DNA G+C content was determined using the HPLC method (Mesbah *et al.*, 1989), and Lambda DNA (Invitrogen) was used as the standard. DNA–DNA hybridization was conducted in microdilution-well plates as described by Ezaki *et al.* (1989). DNA–DNA relatedness was determined using the colorimetric method (Verlander, 1992).

In order to determine the symbiotic properties of strain L6-16T, kidney bean (*Phaseolus vulgaris*) was used as the plant host. Seeds were surface sterilized with 70% ethanol for 10 min and subsequently with absolute ethanol for 5 min in test tubes. The seeds were removed from the tubes, dried on sterilized filter paper and germinated on 0.7% agar plates. Two-day-old seedlings were used for inoculation with strain L6-16T. Seedlings that were inoculated with water and *Rhizobium tropici* CIAT 899T were used as the negative and positive control group, respectively. Plants were grown in closed cylindrical crystal-clear bottles (6.5 × 15 cm) containing sterilized vermiculite moistened with nitrogen-free medium according to the method described by Vincent (1970) and Zhang *et al.* (2011b). Formation of root nodules was examined after 6 weeks of cultivation. Growth conditions used in this study were 16 h/8 h (light/dark) photoperiod and 23 ± 2 °C. The presence of the nodC and nifH genes was examined using primers and temperature profiles described by Laguerre *et al.* (2001).

Strain L6-16T was isolated from *Lemna aequinoctialis* on 1/10 strength TSA medium. Pairwise alignment analysis of the partial 16S rRNA gene sequence of strain L6-16T using the EzTaxon server (Kim *et al.*, 2012) revealed that strain L6-16T was potentially a member of the genus *Rhizobium*. Based on phylogenetic analysis of nearly complete 16S rRNA
gene sequences, the highest sequence similarity value (98.3%) was observed between strain L6-16T and Rhizobium tarimense PL-41T. By contrast, sequence similarity levels between strain L6-16T and other recognized species of the genus Rhizobium species were less than 97.2%. These levels of sequence similarity were relatively low and indicated that strain L6-16T may represent a novel species of the genus Rhizobium (Stackebrandt & Ebers, 2006). Additionally, the phylogenetic tree that was reconstructed using the maximum-likelihood method revealed that strain L6-16T formed a clade with R. tarimense PL-41T. This was significantly supported by a high bootstrap value of 98% (Fig. 1). Additionally, the phylogenetic trees obtained from the maximum-parsimony and neighbour-joining methods consistently supported the relationship between strain L6-16T and R. tarimense PL-41T with bootstrap support levels at 89% and 99%, respectively (Figs S1 and S2, available in the online Supplementary Material).

Horizontal gene transfer and genetic recombination of the rRNA genes in members of the genus Rhizobium have been observed previously Zhang et al. (2011b). Housekeeping genes have been used to confirm classification of members in this genus. Partial sequences of the housekeeping genes recA and atpD of strain L6-16T and other species of the genus Rhizobium were used for phylogenetic analyses. The partial recA gene sequence of strain L6-16T displayed the highest sequence similarity (84.2%) with that of Rhizobium pseudoryzae J3-A127T. The phylogenetic tree reconstructed

**Fig. 1.** Phylogenetic tree reconstructed with the maximum-likelihood method based on nearly complete 16S rRNA gene sequences (1388 bp) of strain L6-16T and type strains of other species of the genus Rhizobium, Bradyrhizobium denitrificans LMG 8443T was used as the outgroup. Bootstrap values are present at tree nodes as the percentage of 1000 replicates. Only values higher than 50% are shown. Bar, 0.01 substitutions per nucleotide position.
based on partial recA sequences using the neighbour-joining and maximum-parsimony methods indicated that strain L6-16T formed a cluster with *R. pseudoryzae* J3-A127T, although this was not significantly supported by the bootstrap analysis (Fig. S3). The partial atpD gene sequence of strain L6-16T showed the highest level of sequence similarity (93.9%) with that of *Rhizobium rosettiformans* W3T. Despite the lack of the significant support by the bootstrap analysis, the phylogenetic tree reconstructed from partial atpD sequences using the neighbour-joining and maximum-likelihood methods showed that strain L6-16T formed a clade with *R. rosettiformans* W3T and *Rhizobium aggregatum* ATCC 43293T (Fig. S4).

The relatively low levels (<93.9%) of housekeeping gene sequence similarity between strain L6-16T and other species of the genus *Rhizobium* suggest that strain L6-16T may represent a separate species of the genus *Rhizobium*. The discrepancy between the phylogenetic positions of strain L6-16T based on the 16S rRNA, recA and atpD genes suggests that these genes of strain L6-16T evolved independently from each other. Similar results were also observed in previous studies. For example, phylogenetic analysis of the 16S rRNA gene indicated that *Rhizobium subbaroanis* JC85T was closely related to *Rhizobium borbori* DN316T, whereas phylogenetic analyses of the glnA and recA genes showed that *R. subbaroanis* JC85T formed a cluster with *Rhizobium huautlense* LMG 18254T and *Rhizobium halophytocola* YC6881T (Ramana et al., 2013). Similarly, the phylogenetic relationship between the type strain of *Rhizobium tubonense* and other recognized species of the genus *Rhizobium* based on the 16S rRNA gene was not in accordance with those obtained from analyses of the recA, atpD and glnII genes (Zhang et al., 2011a).

Cells of strain L6-16T were Gram-stain-negative with a rod shape. Colonies of strain L6-16T were pearl white when grown on YMA medium for 48 h at 30 °C. Monopolar flagella were observed using transmission electron microscopy (Fig. S5). Other phenotypic characteristics of strain L6-16T were also determined. *R. tarimense* NRRL B-59556T, *R. pseudoryzae* J3-A127T and *R. rosettiformans* DSM-26376T were used as reference strains because of their close phylogenetic relationship with strain L6-16T. Distinct phenotypic characteristics between strain L6-16T and the reference strains are shown in Table 1. In contrast to the reference strains, strain L6-16T utilized cellulose, d-galactose, melibiose, trehalose, L-arginine, L-arabinose, adonitol, arabitol, myo-inositol and salicin as sole carbon sources. Strain L6-16T did not grow in YM medium containing 2% (w/v) NaCl while all reference strains were able to tolerate this level of NaCl. Additionally, strain L6-16T was resistant to penicillin G while all reference strains were susceptible to this antibiotic. These characteristics clearly distinguished strain L6-16T from the reference strains.

The analysis of cellular fatty acids has been commonly used in bacterial identification. The results obtained in our study showed that the predominant cellular fatty acids of strain L6-16T were C18:1ω7c and/or C18:1ω6c (summed feature 8; 79.5%) (Table S1). This is consistent with a previous study demonstrating that the presence of C18:1ω7c and/or C18:1ω6c fatty acids (summed feature 8) is common among nodulating bacteria (Tighe et al., 2000). Additionally, C18:1ω7c and/or C18:1ω6c fatty acids (summed feature 8) were found predominantly in all of the reference strains. The differences between the fatty acid profile of strain L6-16T and those of the reference strains were mainly observed in minor constituents. For example, C19:0 cyclo ω8c was absent in strain L6-16T and *R. rosettiformans* DSM-26376T but present in *R. tarimense* NRRL B-59556T (2.6%) and *R. pseudoryzae* J3-A127T (3.3%). C16:1ω7c/C16:1ω6c fatty acids (summed feature 3) were detected in strain L6-16T, *R. tarimense* NRRL B-59556T and *R. pseudoryzae* J3-A127T but were not detected in *R. rosettiformans* DSM-26376T. Other fatty acids of strain L6-16T and the reference strains are listed in Table S1. On the basis of cellular fatty acid profiles, strain L6-16T was clearly distinguishable from the reference strains.

The DNA G+C content of strain L6-16T was 58.1 mol% (Tm). This was consistent with the DNA G+C content range of the genus *Rhizobium* previously reported as 57–66 mol% (Tm) (Young et al., 2001). Levels of DNA–DNA relatedness between strain L6-16T and *R. tarimense* NRRL B-59556T (38.3 ± 0.8%), *R. pseudoryzae* J3-A127T (12.3 ± 0.6%) and *R. rosettiformans* DSM-26376T (6.9 ± 0.4%) were below 70%, which has been accepted as the cut-off value for assigning different bacterial strains to the same species (Wayne et al., 1987). This result clearly indicated that strain L6-16T was distinguishable from phylogenetically related species.

The presence of the *nodC* and *nifH* genes was not detected in strain L6-16T. Additionally, a nodulation test was performed

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<th>Characteristic</th>
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<th>2</th>
<th>3</th>
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<td>d-Galactose</td>
<td>+</td>
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<td>+</td>
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<td>Trehalose</td>
<td>+</td>
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<td>L-Arabinose</td>
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<td>L-Asparagine</td>
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<td>Adonitol</td>
<td>+</td>
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<td>Arabitol</td>
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<td>Dulcitol</td>
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<tr>
<td>myo-Inositol</td>
<td>+</td>
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<td>Salicin</td>
<td>+</td>
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<td>Growth with 2% NaCl</td>
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<td>Resistance to:</td>
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<td>Penicillin G (10 U)</td>
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<td>Streptomycin (10 µg)</td>
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Table 1. Distinct phenotypic characteristics of strain L6-16T and the reference strains

Strains: 1, L6-16T; 2, *R. tarimense* NRRL B-59556T; 3, *R. pseudoryzae* J3-A127T; 4, *R. rosettiformans* DSM-26376T. All data were obtained from this study. +, Positive; –, negative.
with kidney bean (P. vulgaris) to examine the symbiotic properties of strain L6-16<sup>T</sup>. After 6 weeks of cultivation, seedlings of the positive control group that was inoculated with R. tropici CIAT 899<sup>T</sup> formed several root nodules. By contrast, seedlings of the negative control group that were inoculated with strain L6-16<sup>T</sup> did not form nodules. This was consistent with the absence of the nodC and nifH genes in strain L6-16<sup>T</sup>.

Description of Rhizobium lemnae sp. nov.

Rhizobium lemnae (lem’nae. N.L. fem. gen. n. lemnae pertaining to Lemna, the genus of the host plant).

Cells are Gram-stain-negative, motile rods with monopolar flagella. Colonies are circular and pearl white when grown on YM agar medium at 30 °C for 2 days. Growth occurs at 20–41 °C. The optimal growth temperature is 30 °C. The pH range for growth is pH 5–11 (optimum pH 7.0). Tolerates NaCl up to 1 % (w/v) (optimum 0.5 %). Tests for catalase and cytochrome oxidase are positive. Urea is hydrolysed but gelatin is not. The following compounds are utilized as sole carbon sources: cellobiose, d-galactose, melibiose, trehalose, L-arginine, L-arabinose, adonitol, arabitol, myo-inositol and salicin. Does not use the following compounds as sole carbon sources: lactose, glycine, L-asparagine, methionine and dulcitol. Resistant to penicillin G. Susceptible to streptomycin and gentamicin. The predominant cellular fatty acids are C<sub>18:1</sub>ω7c and/or C<sub>18:1</sub>ω6c (summed feature 8).

The type strain is L6-16<sup>T</sup> (=NBRC 109339<sup>T</sup> = BCC 55143<sup>T</sup>), which was isolated from the whole plant of *Lemna aequinoctialis*. The DNA G+C content of the type strain is 58.1 mol% (T<sub>m</sub>).

Acknowledgements

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References


