Rhizobium paknamense sp. nov., isolated from lesser duckweeds (*Lemna aequinoctialis*)

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A Gram-stain-negative, rod-shaped bacterium was isolated and designated strain L6-8^T during a study of endophytic bacterial communities in lesser duckweed (*Lemna aequinoctialis*). Cells of strain L6-8^T were motile with peritrichous flagella. The analysis of the nearly complete 16S rRNA gene sequence indicated that strain L6-8^T was phylogenetically related to species of the genus *Rhizobium*. Its closest relatives were *Rhizobium borbori* DN316^T (97.6 %), *Rhizobium oryzae* Alt 505^T (97.3 %) and *Rhizobium pseudoryzae* J3-A127^T (97.0 %). The sequence similarity analysis of housekeeping genes *recA*, *glnII*, *atpD* and *gyrB* showed low levels of sequence similarity (<91.5 %) between strain L6-8^T and other species of the genus *Rhizobium* with validly published names. The pH range for growth was 4.0–9.0 (optimum 6.0–7.0), and the temperature range for growth was 20–45 °C (optimum 30 °C). Strain L6-8^T tolerated NaCl up to 2 % (w/v) (optimum 1 % NaCl). The predominant components of cellular fatty acids were $C_{19:0}$ cyclo ω8*c* (31.32 %), summed feature 8 ($C_{18:1}\omega$7*c* and/or $C_{18:1}\omega$6*c*; 25.39 %) and $C_{16:0}$ (12.03 %). The DNA G+C content of strain L6-8^T was 60.4 mol% (T_m). *nodC* and *nifH* were not amplified in strain L6-8^T. DNA–DNA relatedness between strain L6-8^T and *R. borbori* DN316^T, *R. oryzae* Alt505^T and *R. pseudoryzae* J3-A127^T was between 11.2 and 18.3 %. Based on the sequence similarity analyses, phenotypic, biochemical and physiological characteristics and DNA–DNA hybridization, strain L6-8^T could be readily distinguished from its closest relatives and represents a novel species of the genus *Rhizobium*, for which the name *Rhizobium paknamense* sp. nov. is proposed. The type strain is L6-8^T (=NBRC 109338^T=BCC 55142^T).

The genus *Rhizobium* was originally described as stem and/or root nodule associated bacteria (Frank, 1889). Species of the genus *Rhizobium* have been traditionally isolated from nodules and the rhizosphere of various leguminous plants. They have been reported to promote plant growth through their nitrogen-fixing activity (Tian *et al.*, 2008; van Berkum *et al.*, 1998; Wang *et al.*, 1998; Zahran, 1999). Aquatic leguminous plants have also been reported to harbour species of the genus *Rhizobium* (Zurdo-Piñeiro *et al.*, 2004). Previous studies showed that novel species of the genus *Rhizobium* can also be found as endophytes colonizing roots of non-leguminous plants including rice, wheat and maize (Peng *et al.*, 2008; Rosenblueth & Martínez-Romero, 2004; Schloter *et al.*, 1997; Zhang *et al.*, 2011b). Their role in promoting growth of non-leguminous plants has also been proposed (Yanni *et al.*, 1997). However, recent studies showed that *Rhizobium skierniewicense* (Puławska *et al.*, 2012a) and *Rhizobium nepotum* (Puławska *et al.*, 2012b) were the cause of the crown gall disease in various plant species. Additionally, *Rhizobium radiobacter* was reported to cause nosocomial infections in humans (Kaselitz *et al.*, 2012).

The family *Lemnaceae*, generally recognized as the duckweed family, consists of 38 species that are classified into five genera including *Lemna*, *Spirodela*, *Landoltia*, *Wolffia* and *Wolffiella* (Wang *et al.*, 2010). Duckweeds are small aquatic plants whose stem and leaves are modified into leaf-like fronds that function in both photosynthesis and vegetative reproduction. Duckweeds are used in basic science research and proposed in various applications. To investigate the diversity of bacterial endophytes in duckweed, *Lemna aequinoctialis*, commonly known as lesser

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene, *recA*, *glnII*, *atpD* and *gyrB* gene sequences of strain L6-8^T are AB733647, AB739029, AB739027, AB739026 and AB739028, respectively.

Five supplementary figures are available with the online version of this paper.

duckweed, was collected from a local pond in Paknam district, Samutprakan Province, Thailand. Healthy-looking plants were rinsed with tap water and surface-sterilized with 10 % sodium hypochlorite solution supplemented with a few drops of Tween 20. Surface-sterilized plants were then washed in sterilized distilled water five times. The surface-sterilization process was confirmed by plating aliquots of distilled water used for the final rinse on 1/10 strength tryptic soy agar (TSA; Himedia). Plants were ground in 5 ml sterilized distilled water with a mortar and a pestle. A ten-times dilution of the suspension was prepared, plated on 1/10 strength TSA plates and incubated at 30 °C for 7 days. Several isolates were randomly chosen and purified by the single-colony streak plate technique.

Genomic DNA of the isolate was prepared according to the method previously described (Araújo *et al.*, 2002) and used for amplification of the 16S rRNA gene using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT- 3′) (Lane, 1991). The following temperature profile was used for amplification: 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 PCR products were sequenced (Macrogen) using universal primers 27F, 1492R, 350F (5′-TACGGGAGGCAGCAG-3′), 780F (5′-GATT-AGATACCCTGGTAG-3′), 1100F (5′-GCAACGAGCGC-AACCC-3′), 350R (5′-CTGCTGCCTCCCGTAG-3′) and 780R (5′-CTACCAGGGTATCTAATCC-3′ (Lane, 1991). Strain L6-8^T was identified as a member of the genus *Rhizobium* based on the pairwise analysis of the 1413 bp 16S rRNA gene sequence using the EzTaxon server (Kim *et al.*, 2012). The sequences of the 16S rRNA genes of other species of the genus *Rhizobium* were obtained from the GenBank database, and multiple alignment analysis was performed using the CLUSTAL W program, version 1.81 (Thompson *et al.*, 1994). The alignment was manually adjusted to eliminate gaps and ambiguous nucleotides. The phylogenetic tree was reconstructed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods in the program MEGA version 5.1 (Tamura *et al.*, 2011). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1980). The confidence levels of the clusters were determined by using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The analysis of 16S rRNA gene sequence similarity indicated that the closest relatives of strain L6-8^T were *Rhizobium borbori* DN316^T, *Rhizobium oryzae* Alt 505^T and *Rhizobium pseudoryzae* J3-A127^T with 97.6 %, 97.3 % and 97.0 % sequence similarity, respectively. The low levels of 16S rRNA gene sequence similarity between strain L6-8^T and its close relatives were below the threshold proposed by Stackebrandt & Ebers (2006) and indicated that strain L6-8^T may represent a distinct species of the genus *Rhizobium*. Phylogenetic trees reconstructed using the neighbour-joining, maximum-parsimony and maximum-likelihood methods consistently showed that strain L6-8^T formed a cluster with *R. borbori* DN316^T (Fig. 1), although this was not significantly supported by the bootstrap analysis.

Cells of strain L6-8^T were Gram-stain-negative bacilli. Cell morphology was observed under a transmission electron microscope (JEM-1230; JEOL). Peritrichous flagella were observed (Fig. S1 available in IJSEM Online). Phenotypic characteristics of strain L6-8 ^{T} were also determined. The type strains of *R. borbori*, *R. oryzae* and *R. pseudoryzae* were used as reference strains because of the high sequence similarity levels based on the 16S rRNA gene and the close phylogenetic relationship to strain L6-8^T. The test for utilization of different carbon sources was carried out using a method described by Gao *et al.* (1994). Tolerance to various NaCl concentrations (1–6 %) and temperatures (20–50 °C) was examined in yeast mannitol agar (YMA) (l^{-1}: 1 g yeast extract, 10 g mannitol, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$.7H2O, 0.05 g NaCl, 0.05 g $CaCl_2$.2H_2O, 0.006 g $FeCl_3$.6 H_2O, 20 g agar; pH7.0) medium. The test for the pH range (pH 4–12) for growth was perfomed using yeast mannitol [YM (YMA medium without agar)] broth. Except for the temperature tests, strain L6-8^T and strains of other reference species were incubated at 30 °C. The results of each test were determined after 48 h of incubation. Nitrate reduction, catalase, urease and cytochrome oxidase activities were detected according to methods previously described (Graham & Parker, 1964; Lindström & Lehtomäki, 1988; MacFaddin, 2000; Skerman, 1967). Differential phenotypic characteristics between strain L6-8^T and the reference species are given in the species description and shown in Table 1. Based on these characteristics, strain L6-8^T was clearly distinct from the reference species. The differences in carbon utilization between strain L6-8^T and its closest relative *R. borbori* DN316^T were that strain L6-8^T did not utilize D-cellobiose, dulcitol, *myo*-inositol, D-salicin, D-xylose, D-mannose, D-galactose, L-arabinose, maltose, L-arginine, L-tyrosine, L-asparagine or Tween 80 but used D-melibiose as the sole carbon source. Strain L6-8^T also differed from *R. borbori* DN316^T with respect to maximum NaCl concentration, pH and temperature for growth.

The presence of the *nodC* and *nifH* genes in strain L6-8^T was investigated using primers and conditions according to a method described by Laguerre *et al.* (2001). The two genes were not amplified by PCR in strain L6-8^T. Several housekeeping genes have been used to confirm classification of species of the genus *Rhizobium* (Zhang *et al.*, 2011a). Partial *recA* (540 bp), *glnII* (586 bp), *atpD* (485 bp) and *gyrB* (643 bp) sequences of strain L6-8^T were amplified using primers and conditions described by Islam *et al.* (2008) and Martens *et al.* (2008). Strain L6-8^T showed the highest *recA* gene sequence similarity to that of *Rhizobium lusitanum* P1-7^T (88.2 %) and less than 87.9 % to type strains of other species of the genus *Rhizobium*. The highest level of *glnII* sequence similarity (90.1 %) was observed between strain L6-8^T and *Rhizobium sullae* IS123^T. The analysis of *atpD* gene sequence similarity

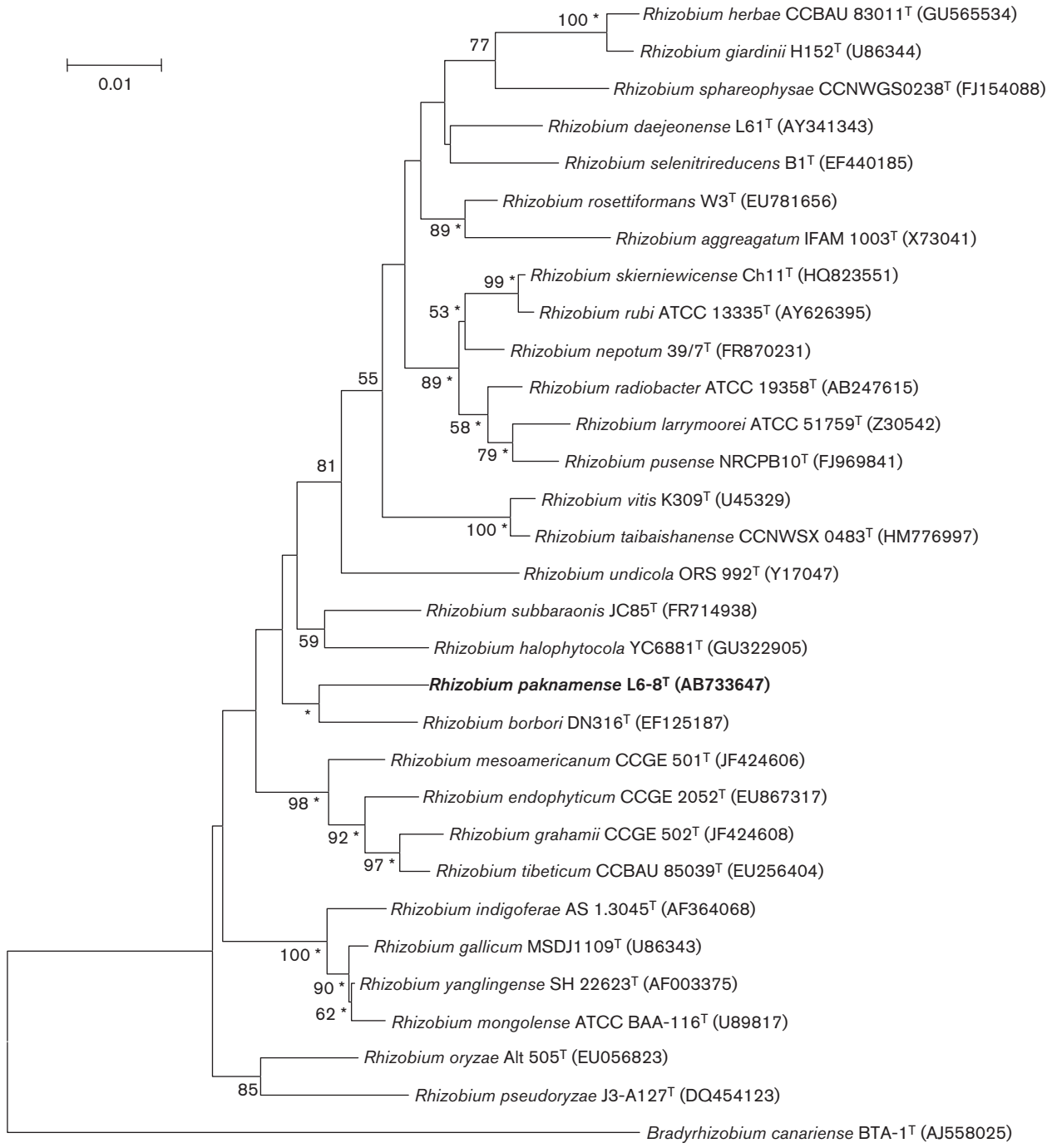

Fig. 1. Phylogenetic tree reconstructed with the neighbour-joining method based on the analysis of nearly complete 16S rRNA sequences of strain L6-8^T and type strains of related species of the genus *Rhizobium*. *Bradyrhizobium canariense* BTA-1^T was used as an outgroup. Asterisks indicate branches of the tree that were also found using maximum-parsimony and maximum-likelihood methods. 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.

displayed the highest similarity level between strain L6-8^T and *Rhizobium undicola* LMG 11875^T (91.1 %). The *gyrB* sequence of strain L6-8^T showed the greatest sequence similarity (91.5 %) to that of *R. undicola* LMG 11875^T and less than 85.9 % to those of type strains of other members of the genus *Rhizobium*. The relatively low levels of housekeeping gene sequence similarity of strain L6-8^T to those of other known species of the genus *Rhizobium*

Table 1. Differential phenotypic characteristics between strain L6-8^T and type strains of phylogenetically related species

Strains: 1, L6-8^T; 2, *R. borbori* DN316^T, 3, *R. oryzae* Alt 505^T; 4, *R. pseudoryzae* J3A-127^T. All data were obtained from this study. +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4
Cytochrome oxidase	−	+	−	+
Utilization as carbon source				
D-Cellobiose	−	+	+	+
Dulcitol	−	+	+	−
myo-inositol	−	+	+	+
D-Salicin	−	+	−	w
D-Melibiose	+	−	+	+
D-Xylose	−	+	+	+
D-Mannose	−	+	+	+
D-Raffinose	−	−	+	−
D -Galactose	−	+	+	+
L-Arabinose	−	+	+	+
Lactose	−	−	+	+
Maltose	−	+	+	+
L-Cysteine	+	+	−	−
L-Arginine	−	+	−	w
L-Tryptophan	w	+	−	−
L-Tyrosine	−	+	−	−
L-Asparagine	−	+	−	w
Glycerol	−	−	+	w
Tween 80	−	w	w	−
Growth at/with:				
2 % NaCl	+	−	−	+
5 % NaCl	−	−	−	+
pH 4	+	−	−	−
pH 10	−	+	+	+
45 °C	+	−	−	−

indicate that strain L6-8^T represents a novel species of the genus *Rhizobium*. Additionally, phylogenetic trees were reconstructed based on *recA*, *atpD*, *gyrB* and concatenated *recA–atpD–gyrB* sequences (Figs S2–S5). The phylogenetic tree based on *recA* sequences showed that strain L6-8^T formed a clade with *R. undicola* LMG 11875^T and *Rhizobium pusense* NRCPB10^T (Fig. S2). The phylogenetic tree based on *gyrB* sequences indicated that strain L6-8^T was clustered with *R. borbori* DN316^T, *R. pseudoryzae* J3-A127^T and *R. undicola* LMG 11875^T (Fig. S4). However, the phylogenetic trees derived from *atpD* and concatenated *recA–atpD–gyrB* sequences consistently showed that strain L6-8^T formed a cluster with *R. borbori* DN316^T, *R. oryzae* Alt 505^T, *R. pseudoryzae* J3-A127^T, *R. undicola* LMG 11875^T and *Bradyrhizobium canariense* CIAT 899^T (Figs S3 and S5). This result supported the close relationship between strain L6-8^T, *R. borbori* DN316^T, *R. oryzae* Alt505^T and *R. pseudoryzae* J3-A127^T based on the sequence similarity analysis of the 16S rRNA gene.

Cellular fatty acid profiles are generally used in bacterial identification. Strain L6-8^T and strains of the reference species were grown in YM medium on a rotary shaker at 30 °C for 7 days and harvested for preparation of freeze-dried cells. Analysis of cellular fatty acid was performed using GLC according to the instructions of the Microbial Identification System (MIDI), Sherlock version 6.0 (Kämpfer & Kroppenstedt, 1996; Sasser, 1990). The predominant cellular fatty acids of strain L6-8^T were $C_{19:0}$ cyclo $\omega 8c$ (31.32 %), summed feature 8 ($C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$; 25.39 %) and $C_{16:0}$ (12.03 %) (Table 2). The presence of summed feature 8 fatty acids in the fatty acid profile of strain L6-8^T was consistent with a previous study demonstrating that these fatty acids were typically found in nodule-forming bacteria (Tighe *et al.*, 2000). Other fatty acids found in strain L6-8^T are listed in Table 2. The fatty acid profile of strain L6-8^T clearly distinguished it from phylogenetically related species.

Chromosomal DNA of strain L6-8^T was extracted according to the method described by Marmur (1962). The DNA G+C content was determined using the HPLC method (Mesbah *et al.*, 1989). Lambda DNA (Invitrogen) was used as the standard for the analysis. The DNA G+C content of strain L6-8^T was 60.4 mol% (T_m). The DNA G+C content of strain L6-8^T is consistent with the range for members of the genus *Rhizobium* that was previously reported as 57–66 mol% (T_m) (Young *et al.*, 2001). DNA–DNA hybridizations between genomic DNA of strain L6-8^T and that of other reference species was performed in microdilution-well plates. DNA–DNA relatedness values (%) were

Table 2. Cellular fatty acids of strain L6-8^T and type strains of phylogenetically related species of the genus *Rhizobium*

Strains: 1, L6-8^T; 2, *R. borbori* DN316^T, 3, *R. oryzae* Alt 505^T; 4, *R. pseudoryzae* J3A-127^T. All data were obtained from this study.

Fatty acid	1	2	3	4
$C_{19:0}$ cyclo $\omega 8c$	31.32	23.81	25.52	13.89
$C_{16:0}$	12.03	20.9	20.26	30.36
$C_{18:0}$ 3-OH	2.67	1.37	0.13	0.44
$C_{18:0}$	2.32	3.22	0.28	0.43
$C_{16:0}$ 3-OH	1.36	0.8	15.37	−
$C_{20:1}\omega 7c$	0.43	0.16	−	−
$C_{18:1}$ 2-OH	0.31	0.19	−	−
$C_{17:0}$ cyclo	0.26	3.98	10.34	9.95
iso-$C_{19:0}$	0.24	−	−	0.1
11-Methyl $C_{18:1}\omega 7c$	0.22	1.33	0.2	−
$C_{17:0}$	0.17	−	0.08	0.12
$C_{18:1}\omega 5c$	0.14	0.17	0.1	−
Summed feature 2*	9.32	2.65	1.29	14.59
Summed feature 3*	5.17	2.53	3.01	1.87
Summed feature 8*	25.39	38.19	13.79	20.94

*Summed features consist of two or more fatty acids that could not be separated by the MIDI system. Summed feature 2 contained $C_{12:0}$ aldehyde, $C_{14:0}$ 3-OH/iso-$C_{16:1}$ I and/or unknown ECL 10.9525; summed feature 3 contained $C_{16:1}\omega 7c$/$C_{16:1}\omega 6c$; summed feature 8 contained $C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$.

obtained using the colorimetric method (Ezaki *et al.*, 1989; Verlander, 1992). The levels of DNA–DNA relatedness between strain L6-8^T and *R. borbori* DN316^T (18.3 ± 1.0 %), *R. oryzae* Alt505 ^{T} (15.7 ± 0.9 %) and *R. pseudoryzae* J3-A127 ^{T} (11.2 ± 0.8 %) were well below the cut-off level of 70 % used for assigning bacterial strains to the same species (Wayne *et al.*, 1987). This confirmed that strain L6-8^T was distinct from phylogenetically related species.

Description of *Rhizobium paknamense* sp. nov.

Rhizobium paknamense (pak.nam.en′se N.L. neut. adj. *paknamense* pertaining to Paknam district, where the type strain was isolated).

Cells are Gram-stain-negative, motile, rods with peritrichous flagella. Colonies are circular, white, semi-translucent and mucilaginous when grown on YMA medium at 30 °C for 2 days. Growth occurs at 20–45 °C. The optimal growth temperature is 30 °C. The pH range for growth is 4.0−9.0 (optimum pH 6.0−7.0). Tolerates NaCl concentration up to 2 % (w/v) (optimum 1 %). Does not reduce nitrate to nitrite. Tests for catalase and cytochrome oxidase are negative. Hydrolyses urea. The following compounds are utilized as sole carbon sources: trehalose, D-melibiose, L-rhamnose, L-cysteine and L-tryptophan. Does not use the following compounds as sole carbon sources: dulcitol, *myo*-inositol, L-arginine, D-salicin, maltose, D-xylose, raffinose, D-galactose, D-mannose, D-cellobiose, L-arabinose, lactose, L-asparagine L-tyrosine, glycerol and Tween 80. The predominant cellular fatty acids are $C_{19:0}$ cyclo $\omega 8c$, summed feature 8 ($C_{18:1}\omega 7c$ and/or $C_{18:1}\omega 6c$) and $C_{16:0}$.

The type strain, L6-8^T (=NBRC 109338^T=BCC 55142^T), was isolated from whole fresh plants of lesser duckweed (*Lemna aequinoctialis*). The DNA G+C content of the type strain is 60.4 mol% (T_m).

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