Labrys neptuniae sp. nov., isolated from root nodules of the aquatic legume *Neptunia oleracea*

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A bacterium designated strain Liujia-146^T was isolated in the Tainan area of southern Taiwan from root nodules of the aquatic legume *Neptunia oleracea*. 16S rRNA gene sequence analysis indicated that strain Liujia-146^T was highly similar to *Labrys monachus* VKM B-1479^T (97.8%) and *Labrys methylaminiphilus* JLW10^T (95.5%) and belonged to the order *Rhizobiales* in the *Alphaproteobacteria*. On the basis of phylogenetic analysis, DNA–DNA hybridization data, physiological and biochemical characteristics and fatty acid compositions, the organism was shown to belong to the genus *Labrys* whilst representing a novel species within this genus. We propose to classify strain Liujia-146^T (=BCRC 17578^T=LMG 23578^T) as the type strain of *Labrys neptuniae* sp. nov.

The pan-tropical mimosoid legume genus *Neptunia* has attracted much interest in the last 15 years, largely because of the aquatic habitat of some of its species and the ability of some of these to form N₂-fixing root nodules on submerged roots (James *et al.*, 1992a, b, 2001; Subba-Rao *et al.*, 1995). A variety of bacteria have been isolated from such nodules, particularly from *Neptunia oleracea* (syn. *N. natans*, *N. prostrata*; <http://www.ildis.org>), including a species closely related to *Rhizobium*, *Allorhizobium undicola* (de Lajudie *et al.*, 1998), and the alphaproteobacterium *Devosia neptuniae* (Rivas *et al.*, 2002, 2003). In addition to these ‘exotic’ bacterial species, more ‘conventional’ rhizobia have also been isolated from *Neptunia* nodules, for example *Rhizobium tropici* strains UPRM8033 and DUS239 from *Neptunia plena* (Zurdo-Piñero *et al.*, 2004). In the present study, we report on a novel non-nodulating strain from the genus *Labrys*, Liujia-146^T, isolated from root nodules of *N. oleracea* growing aquatically in Taiwan. *Labrys* is a genus of budding bacteria, first described by Vasilyeva & Semenov (1984). At present, it consists of two species, *Labrys*

monachus (Vasilyeva & Semenov, 1984) and *Labrys methylaminiphilus* (Miller *et al.*, 2005). Cells of *L. monachus* possess triangular radial symmetry (Vasilyeva & Semenov, 1984) and *L. methylaminiphilus* is a facultative methylotroph (Miller *et al.*, 2005). Comparisons of their 16S rRNA gene sequences indicate that *L. monachus* and *L. methylaminiphilus* belong to the *Alphaproteobacteria* (Fritz *et al.*, 2004; Miller *et al.*, 2005).

Root nodules were collected from *N. oleracea* growing in a freshwater pond at Tainan County in southern Taiwan. They were immersed in 75% ethanol for 10 s, sterilized in 0.1% (w/v) mercuric chloride for 10 min and then washed six times with sterile distilled water. Individual nodules were crushed and streaked onto yeast extract-mannitol (YEM) agar (Vincent, 1970) and incubated at 28 °C. The majority of isolates (>95%) were identified as *A. undicola*. However, a bacterial strain with a colony morphology different from that of *A. undicola* was also recovered. This was designated Liujia-146^T. Type strains of *L. monachus* (DSM 5896^T) and *L. methylaminiphilus* (DSM 16812^T) were obtained from the DSMZ.

The pH range for growth was determined by measuring the optical density (wavelength 595 nm) of cultures grown in YEM medium with pH ranging from 4 to 10, adjusted with appropriate biological buffers (Chung *et al.*, 1995).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Liujia-146^T is DQ417335.

Fatty acid compositions and whole-cell protein profiles of strain Liujia-146^T and related strains are available as supplementary material in IJSEM Online.

Fig. 1. Scanning electron micrograph of a cell of strain Liujia-146^T. Bar, 1 μm.

Anaerobic cultivation was performed on YEM medium under the Oxoid AnaeroGen system. Strain Liujia-146^T formed visible colonies that were circular, convex, opaque and mucoid with entire edges. The colony diameter was approximately 1.0–2.0 mm on YEM agar after 48 h of incubation at 28 °C. Strain Liujia-146^T grew well at temperatures ranging from 25 to 35 °C and at a pH between 4 and 10; optimal growth conditions were 28–30 °C and pH 7.0–8.0. Strain Liujia-146^T did not require additional growth factors, although addition of yeast extract was found to stimulate growth. It did not grow over 120 h of incubation at 28 °C under anaerobic conditions, suggesting that it is an aerobic bacterium.

Cell morphology was observed under a light microscope and by scanning electron microscopy. Motility of cells was examined by the hanging drop method. Gram Stain Set S (Difco) and Indian ink (Difco) were used to perform Gram and capsule staining. Strain Liujia-146^T is a Gram-negative, non-motile, non-spore-forming, capsulated, rod-shaped bacterium, 0.7–0.9 μm in diameter and 1.2–1.5 μm in length (Fig. 1). Cells are found singly or in pairs and were seen to reproduce by budding. Capsule formation, observed by electron microscopy, was also visualized with Indian ink by light microscopy.

Amplification and sequence analysis of the 16S rRNA gene were performed as described elsewhere (Chen *et al.*, 2001). Multiple-sequence alignment comparing strain Liujia-146^T and its closest relatives was performed using BioEdit software (Hall, 1999). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining and maximum-parsimony methods were determined by using bootstrap values based on 1000 replications. Comparison of the 16S rRNA gene sequence of strain Liujia-146^T with available 16S rRNA gene sequences in public databases revealed that strain Liujia-146^T belonged to the order *Rhizobiales* of the *Alphaproteobacteria*. The highest similarity values were obtained from the comparison with *L. monachus* VKM B-1479^T (97.8 % similarity) and *L. methylaminiphilus* JLW10^T (95.5 %). The two type strains of

Labrys species, VKM B-1479^T and JLW10^T, and strain Liujia-146^T were found to form a well-separated, monophyletic group (Fig. 2). The 16S rRNA gene sequence similarity of strain Liujia-146^T to other established bacterial species within the *Alphaproteobacteria* was less than 95 %. Recently, Lee *et al.* (2005) proposed a novel family *Xanthobacteraceae* of the order *Rhizobiales* to include the genera *Labrys*, *Azorhizobium*, *Ancylobacter*, *Starkeya* and *Xanthobacter*. However, our phylogenetic analysis based on neighbour-joining and maximum-parsimony methods did not support the affiliation of *Labrys* species within the family *Xanthobacteraceae*, which was based on only one species, *L. methylaminiphilus*.

DNA–DNA hybridization experiments were performed with strain Liujia-146^T and the two type strains of *Labrys* species using the method described by Ezaki *et al.* (1989), with data from experiments performed in triplicate indicating that the DNA–DNA binding level of strain Liujia-146^T with *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T was only 18.6 (±5.1) % and 41.0 (±7.6) %, respectively. The DNA G+C content of strain Liujia-146^T was determined in triplicate using the method described by Mesbah *et al.* (1989) and was found to be 62.7 (±1.7) mol%.

For biochemical characterization, the API 20NE, API ZYM (bioMérieux) and Microlog GN2 (Biolog) systems were used according to the manufacturers' instructions. Additionally, sensitivity to antibiotics was examined by spreading cells (0.5 McFarland) onto YEM agar and placing onto them discs (Difco) containing the following individual antibiotics: ampicillin (10 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), novobiocin (30 μg), rifampicin (5 μg), penicillin G (10 U), streptomycin (10 μg) and tetracycline (30 μg). Results of biochemical characterization and antibiotic sensitivity are given in the species description.

Chemotaxonomic differentiation of strain Liujia-146^T from its closest phylogenetic neighbours was examined using several approaches. For analysis of protein electrophoretic patterns, preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot *et al.* (1994). The whole-cell protein profile of strain Liujia-146^T could be differentiated clearly from those of *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T (see Supplementary Fig. S1 available in IJSEM Online). For fatty acid methyl ester analysis, cell culture was harvested after an incubation period of 48 h at 28 °C; fatty acid methyl esters were then prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The major cellular fatty acids of strain Liujia-146^T were 16:0, 18:1ω7c and 19:0 cyclo, as found with *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T (Miller *et al.*, 2005). However, strain Liujia-146^T could again be clearly distinguished from *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T by its significantly larger relative amount of 18:1ω7c (68.8 %), coupled with the

Fig. 2. Neighbour-joining phylogenetic tree of strain Liujia-146^T and related bacteria based on 16S rRNA gene sequence comparisons. Numbers at nodes are percentage bootstrap values based on 1000 resampled datasets; only values above 50 % are given. Accession numbers are given in parentheses. Bar, 1 % sequence dissimilarity.

smaller relative amount of 19:0 cyclo (7.8 %) and the absence of detectable amounts of 20:1 ω 9 (Table 1 and Supplementary Table S1 in IJSEM Online).

Plant cultivation and nodulation tests were carried out as described previously (Chen *et al.*, 2003). Plants of *N. oleracea* and *Macroptilium atropurpureum* were harvested at 45 days after inoculation with strain Liujia-146^T, and no nodules had formed by that time. This indicates that bacteria isolated from nodules are not necessarily able to induce nodulation.

Detailed comparison of the physiological and biochemical characteristics of strain Liujia-146^T, *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T is shown in Table 1. Strain Liujia-146^T could be distinguished clearly from *L. monachus* VKM B-1479^T by many characters, especially regarding the capability for nitrite reduction, vitamin requirement for growth and assimilation of a variety of carbon substrates. Strain Liujia-146^T could also be distinguished clearly from *L. methylaminiphilus* JLW10^T by some enzyme activities (such as oxidase, catalase, arginine dihydrolase, β -galactosidase and aesculin hydrolysis) and assimilation of some carbon substrates. In addition to physiological and biochemical evidence, fatty acid compositions, whole-cell protein profiles and

DNA–DNA hybridization tests could also be used to distinguish strain Liujia-146^T from the nearest species, *L. monachus* and *L. methylaminiphilus*. Therefore, based on phenotypic and phylogenetic criteria, we are of the opinion that strain Liujia-146^T merits assignment to a novel species within the genus *Labrys*, for which the name *Labrys neptuniae* sp. nov. is proposed.

Description of *Labrys neptuniae* sp. nov.

Labrys neptuniae (nep.tu'ni.ae. N.L. gen. n. *neptuniae* of *Neptunia*, named because the type strain was isolated from *Neptunia oleracea*).

Cells are Gram-negative, non-motile, non-spore-forming, capsulated, rod-shaped and multiply by budding. They are 0.7–0.9 μ m in diameter and 1.2–1.5 μ m in length. Growth is evident at temperatures between 15 and 35 °C and at pH 4.0–10.0; optimum growth is displayed at 28–30 °C and pH 7.0–8.0. The following characters (API 20NE) are positive for the type strain: nitrate reduction, urease, aesculin hydrolysis, β -galactosidase and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate and malate. Negative results are displayed for oxidase, indole production, glucose fermentation, arginine dihydrolase, gelatin hydrolysis and assimilation

Table 1. Comparison of phenotypic and biochemical characteristics between strain Liujia-146^T, *L. monachus* and *L. methylaminiphilus*

Strains: 1, *L. monachus* VKM B-1479^T; 2, *L. methylaminiphilus* JLW10^T; 3, strain Liujia-146^T (*L. neptuniae* sp. nov.). +, Positive response; −, negative response; W, weak reaction. Results were acquired in the present study, with the exception of the DNA G+C content and major fatty acid composition data for *L. monachus* VKM B-1479^T and *L. methylaminiphilus* JLW10^T, which were obtained from Miller *et al.* (2005). The following characters were positive for all strains: reduction of nitrate to nitrite and assimilation of arabinose, arabitol, i-erythritol, D-fructose, L-fucose, D-galactose, α-D-glucose, D-mannitol, D-mannose, D-psicose, methyl pyruvate, monomethyl succinate, β-hydroxybutyric acid, succinamic acid, DL-lactic acid, alaninamide, D-alanine, L-alanine, L-alanyl glycine and L-proline. All strains were negative for assimilation of D-lactose, maltose, sucrose, turanose, citric acid, D-glucuronic acid, itaconic acid, α-ketoglutaric acid, α-ketovaleric acid, α-saccharic acid and α-sebacic acid.

Characteristic	1	2	3
Vitamin requirement for growth	+	−	−
Reduction of nitrate to nitrogen	−	+	+
Oxidase	−	+	−
Catalase	W	+	W
Arginine dihydrolase	−	+	−
β-Galactosidase	−	−	+
Aesculin hydrolysis	−	−	+
Assimilation of:			
Tween 80	−	−	+
D-Raffinose	−	−	+
D-Trehalose	−	−	+
Gentiobiose	−	+	+
myo-Inositol	−	+	+
Melibiose	−	+	+
p-Hydroxyphenylacetic acid	−	−	+
L-Phenylalanine	−	+	−
Putrescine	−	−	+
2-Aminoethanol	−	−	+
Glycyl L-aspartic acid	−	+	−
Major fatty acids (%):			
16:0	19.7	17.7	12.9
18:1ω7c	2.9	32.8	68.8
19:0 cyclo	40.4	49.4	7.8
20:1ω9	11.9	−	−
DNA G+C content (mol%)	68	65.7	62.7

of maltose, caprate, adipate, citrate and phenylacetate. Positive results (API ZYM) are seen for activities of alkaline phosphatase, C4 esterase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase and negative results are obtained for C8 lipase, C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, α-glucosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The following carbon sources are oxidized

(positive result with the Biolog GN2 system): glycogen, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, myo-inositol, lactulose, D-mannitol, D-mannose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, D-trehalose, xylitol, methyl pyruvate, monomethyl succinate, acetic acid, cis-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketobutyric acid, DL-lactate, malonic acid, propionic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, putrescine, 2-aminoethanol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. The type strain can not oxidize α-cyclodextrin, dextrin, α-D-lactose, maltose, sucrose, turanose, citrate, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, itaconic acid, α-ketoglutaric acid, α-ketovaleric acid, quinic acid, D-saccharic acid, sebacic acid, glycyl L-aspartic acid, L-phenylalanine, D-serine, phenylethylamine or 2,3-butanediol. The type strain is resistant to ampicillin, chloramphenicol, nalidixic acid, penicillin G and rifampicin and sensitive to gentamicin, kanamycin, novobiocin, streptomycin and tetracycline. The major fatty acid components are 18:1ω7c, 16:0 and 19:0 cyclo. The DNA G+C content of the type strain is 62.7 (±1.7) mol%.

The type strain, Liujia-146^T (=BCRC 17578^T=LMG 23578^T), was isolated from root nodules of *Neptunia oleracea*, an aquatic legume, growing in a freshwater pond located in Tainan County in southern Taiwan.

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