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A bacterium designated strain Liujia-146ᵀ 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-146ᵀ was highly similar to Labrys monachus VKM B-1479ᵀ (97.8%) and Labrys methylaminiphilus JLW10ᵀ (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-146ᵀ (=BCRC 17578ᵀ =LMG 23578ᵀ) 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-Piñeiro et al., 2004). In the present study, we report on a novel non-nodulating strain from the genus Labrys, Liujia-146ᵀ, 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-146ᵀ. Type strains of L. monachus (DSM 5896ᵀ) and L. methylaminiphilus (DSM 16812ᵀ) 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-146ᵀ is D0417335.

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

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

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Anaerobic cultivation was performed on YEM medium under the Oxoid AnaeroGen system. Strain Liujia-146T 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-146T 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-146T 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 examinations by the hanging drop method. Gram Stain Set S was prepared, separated and identified according to the instructions of the Microbial Identification System (MIDI; Microlog). The major cellular fatty acids of strain Liujia-146T were 16 : 0, 18 : 1 v7c and 19 : 0 cyclo, as found with L. methylaminophilus JLW10T. Bar, 1 μm.

Amplification and sequence analysis of the 16S rRNA gene were performed as described elsewhere (Chen et al., 2001). Multiple-sequence alignment comparing strain Liujia-146T 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-146T with available 16S rRNA gene sequences in public databases revealed that strain Liujia-146T belonged to the order Rhizobiales of the Alphaproteobacteria. The highest similarity values were obtained from the comparison with L. monachus VKM B-1479T (97.8 % similarity) and L. methylaminophilus JLW10T (95.5 %). The two type strains of Labrys species, VKM B-1479T and JLW10T, and strain Liujia-146T were found to form a well-separated, monophyletic group (Fig. 2). The 16S rRNA gene sequence similarity of strain Liujia-146T 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. methylaminophilus.

DNA–DNA hybridization experiments were performed with strain Liujia-146T 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-146T with L. monachus VKM B-1479T and L. methylaminophilus JLW10T was only 18.6 (±5.1) % and 41.0 (±7.6) %, respectively. The DNA G+C content of strain Liujia-146T 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 μU), 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-146T 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-146T could be differentiated clearly from those of L. monachus VKM B-1479T and L. methylaminophilus JLW10T (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-146T were 16:0, 18:1ω7c and 19:0 Cyclo, as found with L. monachus VKM B-1479T and L. methylaminophilus JLW10T (Miller et al., 2005). However, strain Liujia-146T could again be clearly distinguished from L. monachus VKM B-1479T and L. methylaminophilus JLW10T by its significantly larger relative amount of 18:1ω7c (68.8 %), coupled with the

Fig. 1. Scanning electron micrograph of a cell of strain Liujia-146T. Bar, 1 μm.
smaller relative amount of 19:0 cyclo (7.8 %) and the absence of detectable amounts of 20:1 \(v_9\) (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, \(\beta\)-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 \(\mu m\) in diameter and 1.2–1.5 \(\mu m\) 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, arginine dihydrolase, \(\beta\)-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.
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, z-chymotrypsin, z-galactosidase, z-glucosidase, 3-glucuronidase, 3-glucosidase, N-acetyl-z-glucosaminidase, 3-mannosidase and 3-fucosidase. The following carbon sources are oxidized (positive result with the Biolog GN2 system): glycogen, Tween 40, Tween 80, N-acetyl-z-galactosamine, N-acetyl-d-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, L-erythritol, D-fructose, L-fucose, z-galactose, z-D-glucose, D-mannitol, D-mannose, D-psicose, methyl pyruvate, monomethyl succinate, z-hydroxybutyric acid, succinic acid, formic acid, z-glucuronide, L-glutamine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl z-glutaminate acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, DL-carnitine, z-aminobutyric acid, uracil acid, inosine, uridine, thymidine, putrescine, 2-aminomethyl, glycerol, DL-z-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. The type strain can not oxidize z-cyclodextrin, dextrin, z-D-lactose, maltose, sucrose, turanose, citrate, D-glucosaminic acid, D-glucuronic acid, z-hydroxybutyric acid, itaconic acid, z-ketoglutaric acid, z-ketolactic acid, quinic acid, D-saccharic acid, sebamic acid, glycol z-aspartic acid, L-phenylalanine, D-serine, phenylethylamine or 2,3-butandiol. 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 : 1o7c, 16 : 0 and 19 : 0 cyclo. The DNA G + C content of the type strain is 62.7 (± 1.7) mol%.

The type strain, Liuji-146T (= BCRC 17578T = LMG 23578T), 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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