Labrys soli sp. nov., isolated from the rhizosphere of ginseng

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In this study, we describe strain DCY64T that was isolated from the rhizosphere of three-year-old Korean ginseng root. Cells were Gram-reaction negative, oxidase- and catalase-positive, strictly aerobic, capsulated, non-motile, non-sporulating and spherical to short rod-shaped. Multiplicative budding cells were produced. Vesicles covered the surface of cells. Phylogenetic analysis placed strain DCY64T within the genus Labrys with the highest similarity to Labrys monachus VKM B-1479T (97.6 % 16S rRNA gene sequence similarity), followed by Labrys okinawensis MAFF 210191T (97.5 %), Labrys miyagiensis G24103T (97.4 %), and Labrys portucalensis F11T (97.0 %). The genomic DNA G+C content was 63 mol%. The presences of summed feature 8 (C18 : 1v7c and/or C18 : 1v6c), C19 : 1cyclo8c and C16 : 0 as major fatty acids; phosphatidylmonomethylethanolamine, phosphatidylglycerol, phosphatidylcholine and diphasphatidylglycerol as major polar lipids; ubiquinone Q-10 as the predominant quinone and sym-homospermidine as the dominant polyamine were found in strain DCY64T. These chemotaxonomic results were in accordance with those of members of the genus Labrys. However, the absence of C16 : 02-OH, C16 : 03-OH and C18 : 12-OH from the fatty acids profile and differences in minor polar lipids and phenotypic characteristics distinguished strain DCY64T from the closest type strains. The discrimination was also supported by unique enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) fingerprints, as well as DNA–DNA hybridization values (≤48 %) between strain DCY64T and related type strains. Therefore, we propose that strain DCY64T represents a novel species of the genus Labrys. The name Labrys soli sp. nov. is proposed, with DCY64T (=KCTC 32173T=JCM 19895T) as the type strain.

First proposed by Vasilyeva & Semenov (1984, 1985), the genus Labrys included the species Labrys monachus. Historically, this genus was established on the basis of cell morphology and DNA–DNA hybridization values. Later, Fritz et al. (2004) studied the phylogenetic affiliation of this genus and placed this genus in the class Alphaproteobacteria. Islam et al. (2007) emended the genus with information on cell morphology, motility, DNA G+C content (range of 61.0–66.0 mol%), major fatty acids (C19 : 0 cyclo8c, C16 : 0, C18 : 0 and C18 : 1v7c) and quinone (Q-10). Subsequently, this genus was further emended by Albert et al. (2010), including that members of the genus are aerobes or facultative anaerobes; diphosphatidylglycerol, phosphatidylmonomethylethanolamine and phosphatidylcholine are major polar lipids; and sym-homospermidine is the major polyamine. At the time of writing, the genus Labrys includes seven species with validly published names isolated from different environments, such as Labrys monachus isolated from silt of a lake (Vasilyeva & Semenov, 1984), Labrys methylaminophilus isolated from a freshwater sediment (Miller et al., 2005), Labrys okinawensis and Labrys miyagiensis isolated from rhizosphere (Islam et al., 2007), Labrys neptuniae isolated from root nodules (Chou et al., 2007), Labrys portucalensis isolated from

Abbreviation: ERIC-PCR, enterobacterial repetitive intergenic consensus sequence PCR.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DCY64T is JX315532.

A supplementary table and two supplementary figures are available with the online Supplementary Material.
sediment (Carvalho et al., 2008) and Labrys wisconsinensis isolated from water (Albert et al., 2010). In this study, we used polyphasic approaches to identify a novel strain designated DCY64T which was isolated from the rhizosphere of ginseng root in Republic of Korea as representing a member of the genus Labrys.

Three-year-old ginseng roots were collected from Gyeryong Mountain, Chungcheong province, Republic of Korea (36° 20′ 36″ N 127° 12′ 22″ E). Rhizosphere soil was obtained by shaking the roots with sterilized saline [0.85 % (w/v) NaCl] in sterile 50 ml conical tubes. Subsequently, the rhizosphere soil suspensions were diluted with sterilized saline using standard dilution methods. The soil suspension was spread on modified Reasoner’s 2A (R2A; MB cell) agar medium (1/5-strength R2A) and then incubated at 30 °C for 3 days. Single colonies were transferred to fresh R2A agar medium three times to ensure purity. The preservation of isolates was performed by keeping cells in 1/5-strength R2A broth containing 30 % (v/v) glycerol at −70 °C.

Amplification, purification and sequencing of the 16S rRNA gene was performed using the universal bacterial primer sets, 27F, 518F, 800R and 1492R (Lane, 1991; Weisburg et al., 1991), by Genotech (Daejeon, Republic of Korea). SeqMan software was used to compile the fragment sequences of the 16S rRNA gene to obtain the nearly complete sequence (1391 nt). To evaluate pairwise similarities, the sequence was compared with sequences on the GenBank database. The phylogenetic affiliation was deduced by neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods using the MEGA 6.0 programme (Tamura et al., 2013) with distance calculated with the gamma model of Tamura & Nei (1993). Bootstrap analysis with 1000 replications was also conducted to evaluate the tree topologies (Felsenstein, 1985). DCY64T shared highest similarity to Labrys monachus VKM B-1479T (97.6 % 16S rRNA gene sequence similarity), followed by Labrys okinawensis MAFF 210191T (97.5 %), Labrys miyagiensis G24103T (97.4 %) and Labrys portucalensis F11T (97.0 %). The three phylogenetic analysis methods (Fig. 1) clearly showed that DCY64T clustered within the group containing members of the genus Labrys. This cluster was supported by 100 % bootstrap value that is consistent with the results of previous studies (Miller et al., 2005; Islam et al., 2007; Chou et al., 2007; Carvalho et al., 2008; Albert et al., 2010). However, DCY64T formed a monophyletic branch in this group. The type strain Labrys monachus ICMP 21795T was obtained from the Japan Collection of Microorganisms, Labrys okinawensis DSM 18385T and Labrys portucalensis DSM 17916T were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell cultures and Labrys miyagiensis KCTC 22027T was obtained from the Korean Collection for Type Cultures, for comparative purposes.

To examine cell morphology, we used a transmission electron microscope (LEO912AB, Carl Zeiss). Transmission electron micrographs were obtained after fixing 24-hour-old suspended cells onto Formvar-carbon coated nickel grids and negative staining with 2 % (w/v) phoshotungstic acid (PTA; pH 7.2). To assess the Gram type of the cells, a Gram staining kit (Sigma-Aldrich) was used. Motility, sporulation and capsule formation were checked by the hanging drop technique, staining with malachite green and staining with Indian ink, respectively (Prescott & Harley, 2001). The ability to grow under anaerobic conditions was tested by incubating bacterial plates (R2A agar) in a BD GasPak EZ anaerobe pouch system with indicator (Becton Dickinson) and an AnaeroPack Rectangular Jar (Mitsubishi Gas Chemical) that contained an Anaer pack-anaero (Mitsubishi Gas Chemical) for 14 days at 30 °C. Oxidase and catalase activities were assessed by adding oxidase reagent (bioMérieux) and 3 % (w/v) H2O2 solution, respectively. The temperature range for growth was checked at 4, 10, 15, 20, 25, 28, 30, 37 and 40 °C in R2A broth for 5 days. Growth in various media was assessed using R2A, nutrient agar (NA; MB cell), trypticase soy agar (TSA; MB cell), Luria–Bertani agar (LB; MB cell) and MacConkey agar (Difco) at 30 °C for 5 days. The pH range for growth was determined by adjustment to pH 4–10 at intervals of 0.5 pH units in R2A broth using the following buffers: citric acid/sodium citrate (pH 4.0–6.0), Na2HPO4/NaH2PO4 (pH 6.0–8.0), Tris/HCl (pH 8.5–9.0) and glycine/NaOH (pH 9.5–10.0) (Gomori, 1955). Salt tolerance was tested in R2A broth medium supplemented with 0–1 % (w/v) NaCl in increments of 0.1 % at 30 °C for 7 days. Hydrolysis of starch, Tween 20, Tween 80, DNA, tyrosine and casein were performed as described by Barrow & Feltham (1993). Physiological and biochemical characteristics were further checked using API 20NE, ID 32GN and API ZYM tests. API tests were performed according to the instructions of the manufacturer (API bioMérieux). For checking antibiotic sensitivity of the isolate, the following antibiotics (μg per disc, except for penicillin G; Oxoid) were tested: carbencillin (100), ceftazolin (30), cefazidime (30), erythromycin (15), lincomycin (15), neomycin (30), novobiocin (30), oleandomycin (15), penicillin G (10 units), rifampicin (5), tetracycline (30) and vancomycin (30). The responses were assayed by the disc diffusion method according to the protocol of Nokhal & Schlegel (1983). The bacterial inocula were spread on Mueller–Hinton agar (Difco) plates. The susceptibility of cells to antibiotics was evaluated after 2 days of incubation at 30 °C under aerobic condition. Production of siderophore and solubilization of phosphate were checked as per methods described by Schwyn & Neilands (1987) and Pikovskaya (1948) with the modification of using Pseudomonas agar F (Difco) medium supplemented with a chrome azurol S complex for production of siderophore
and commercial Pikovskaya’s Broth medium (HiMedia) for the phosphate solubilizing test.

Cells were spherical to short-rod shaped (Fig. 2), which is different from the published description of L. monachus JCM 21795T which has flat or triangular cells with prosthecae in two of the three corners, but similar to those of L. okinawensis DSM 18385T and L. miyagiensis KCTC 22027T. Cells appeared singly (Fig. 2a), or in pairs (Fig. 2b) and multiplied by budding (Fig. 2c, d). The ability of strain DCY64T to undergo budding is also a typical characteristic of members of the genus Labrys. Interestingly, on the surface of cells we observed vesicles that were also present in outer membrane of cells of Labrys neptuniae Liuja-146T (Chou et al., 2007; but which the authors did not describe). Other physiological and biochemical properties of strain DCY64T are included in the species description and the differences in biochemical characteristics of strain DCY64T from closely related and concurrently tested type strains of species of the genus Labrys are listed in Table 1. DCY64T differed from the reference type strains with respect to its ability to hydrolyse Tween 80. Compared with the most closely related type strain, L. monachus JCM 21795T, strain DCY64T was able to utilize starch, N-acetylglucosamine, potassium gluconate, sodium acetate, L-alanine, potassium 5-gluconate and L-histidine but was not able to reduce nitrate to nitrite or to degrade urea.

For fatty acids analysis, cell biomass of strain DCY64T and four reference type strains was collected from cells grown in R2A agar at 30°C for 18–24 h. Fatty acids were extracted, methylated using the standard protocol of MIDI (Sherlock Microbial Identification System) as described by Sasser (1990) and then separated by gas chromatography (GC 6890, Agilent). Identification of the methyl esters was conducted by using the TSBA library (version 6.1). Quinones were extracted from 100 mg freeze-dried cells and analysed using HPLC (Hiraishi et al., 1996). Polar lipids of DCY64T and the three reference type strains were extracted and analysed by two dimensional TLC as described by Minnikin et al. (1984). Polyamine profiles of strain DY64T and the most closely related type strain, L. monachus JCM 21795T, were generated in parallel as described in the literature (Busse & Auling 1988; Taibi et al., 2000). HPLC systems analyses for detection of ubiquinone and polyamines were set up as previously (Nguyen et al., 2015).
The fatty acids profile of strain DCY64\textsuperscript{T} included a predominant amount of summed feature 8 (C\textsubscript{18:1}ω7\textsubscript{c} and/or C\textsubscript{18:1}ω6\textsubscript{c}; 61.4\%); major amounts of C\textsubscript{19:1}\text{cyclo} ω8\textsubscript{c} (16.8\%) and C\textsubscript{16:0} (13.6\%); and minor amounts of summed feature 2 (comprising C\textsubscript{12:0} aldehyde and/or unknown fatty acid of equivalent chain-length 10.928; 4.0\%), C\textsubscript{18:0} 3-OH (2.2\%) and C\textsubscript{18:0} (2.0\%) (Table S1 available in the online Supplementary Material). This profile slightly differed with respect to those of reference type strains in this study by the absence of C\textsubscript{16:0} 2-OH, C\textsubscript{16:0} 3-OH and C\textsubscript{18:1} 2-OH. The differences in relative amounts of summed feature 8 (C\textsubscript{18:1}ω7\textsubscript{c} and/or C\textsubscript{18:1}ω6\textsubscript{c}) and C\textsubscript{19:1} cyclo ω8\textsubscript{c} between strain DCY64\textsuperscript{T} and reference type strains were not considered to be of relevance for discrimination between species as described by Albert et al. (2010). The quinone systems contained predominantly ubiquinone Q-10 (98.5\%) and trace amounts of Q-11 (1.5\%). The major fatty acids and quinone of DCY64\textsuperscript{T} are in good agreement with those of member of the genus \textit{Labrys} (Islam et al., 2007). The polar lipids patterns of DCY64\textsuperscript{T} and three reference type strains in this study are consistent with those described as part of a previous study by Albert et al. (2010). Major amounts of phosphatidylmonomethylethanolamine, phosphatidylglycerol, phosphatidylycerine and diphosphatidyglycerol, minor to trace amounts of phosphatidyldethanolamine, phosphatidyldimethylethanolamine, two unidentified aminolipids, three unidentified aminophospholipids, four unidentified phospholipids and two unidentified polar lipids were detected in the polar lipids pattern of strain DCY64\textsuperscript{T} (Fig. S1). Strain DCY64\textsuperscript{T} could be discriminated from the three reference type strains by the presence of an unidentified polar lipid and two aminophospholipids. The polyamine profiles of strain DCY64\textsuperscript{T} and \textit{L. monachus} JCM 21795\textsuperscript{T} were similar with major amounts of sym-homospermidine, moderate amounts of putrescine and minor amounts of spermidine.

\textbf{Fig. 2.} Transmission electron micrographs of cells of strain DCY64\textsuperscript{T} grown on R2A agar medium for 24 h at 30 °C, negatively stained with phosphotungstic acid. Cells occurred singly (a) or in pairs (b) and multiplied by budding (c), (d). Arrows indicate vesicles. Bars, (a–c) 200 nm; (d) 500 nm.
This polyamine pattern has been shown to occur in the profile of species of the genus Labrys (Albert et al., 2010).

The genomic DNA of strains for molecular analysis was prepared using Exgene Cell SV mini-kit (Gene All Biotechnology). For analysis of DNA G+C content, after denaturation of genomic DNA with boiling water, ssDNA was degraded with P1 nuclease enzyme and the phosphate group was split out from the nucleotide with alkaline phosphatase (Mesbah et al., 1989). The nucleosides were detected by HPLC (NS-4000, Futecs) using an YM-C Triart C18 (250 × 4.6 mm, 5 μm). Elution was performed with a mixture of 25 mM (NH4)2HPO4/acetoni-trile (20/1, v/v) at a flow rate of 1.0 ml min⁻¹. Detection was performed by UV absorption at a wavelength of 270 nm.

The genomic DNA of Escherichia coli strain B (D4889, Sigma-Aldrich) was used as a standard. Enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) fingerprinting using the primers ERIC-1R and ERIC-2 was carried out as described by Versalovic et al. (1991) to compare strain DCY64T with reference type strains. ERIC-PCR was performed with two replicates for each strain. Amplicons were separated by electrophoresis on a 1.5% (w/v) agarose gel containing ethidium bromide and at 75 V for 55 min. The agarose gel was visualized under a UV gel imager. To confirm the difference between strain DCY64T and the closely related species, DNA–DNA hybridization was carried out. Hybridization experiments were conducted fluorometrically using photobiotin-labelled DNA probe of strain DCY64T in microplate wells as reported by Ezaki et al. (1989). The hybridization temperature was 51 °C. The experiment was performed with five replications for each sample. The highest and lowest values obtained from each sample were excluded and the mean of the remaining three values were used to calculate DNA–DNA relatedness.

The genomic DNA G + C content of strain DCY64T was 63 mol%, which corresponds with the DNA G + C content of the members of the genus Labrys, which ranges between 61.0 and 66.0 mol% (Islam et al., 2007). The ERIC-PCR fingerprint technique has been used and demonstrated successfully to discriminate among strains of species of the genera Campylobacter (Mouwen et al., 2005), Aeromonas (Figueras et al., 2006) and Lactobacillus (Stephenson et al., 2009) by analysis of individual electrophoretic profiles obtained from PCR products. In this study, ERIC-PCR generated a complex of fingerprint upon electrophoresis, with bands ranging mainly from 200 bp to 1500 bp (Fig. S2). The four strains generated different and distinctive band patterns, thus they were considered to be different.

**Description of Labrys soli sp. nov.**

*Labrys* soli (so’li. L. neut. gen. n. soli of soil, the source of the type strain).

Negative for Gram-reaction, positive for catalase and oxidase activity, strictly aerobic, capsulated, non-motile and non-sporulating. Cells are spherical to short-rod-shaped, 2.2–3.5 μm in length. Cells occur singly, or in pairs and multiply by budding. Vesicles are observed on the surface of cells. Colonies are white, circular, flat, opaque and entire with a diameter of 0.5–1.5 mm after incubation on R2A for 3 days. Growth is observed at 25–30 °C (optimum, 30 °C), at pH 4.5–9.5 (optimum, pH 6.5–7.0) and at 0–0.5% (w/v) NaCl.
The fatty acids profile includes a predominant amount of summed feature 8 (C18:1ω7c and/or C18:1ω6c) and major amounts of C19:1 cyclo ω8c and C16:0. The major polar lipids are phosphatidylmonomethylethanolamine, phosphatidylglycerol, phosphatidylcholine and diphosphatidylglycerol. The predominant ubiquinone is Q-10. The dominant polyamine is sym-homospermidine.

The type strain, DCY64T (≡KCTC 32173T =JCM 19895T), was isolated from rhizosphere of Korean ginseng. The genomic DNA G+C content of the type strain is 63 mol%.

Acknowledgements

This research was supported by a grant from the Korea Institute of Planning & Evaluation for Technology in Food, Agriculture, Forestry & Fisheries (KIPET no. 313038-03-2-SB010), and also supported by the Next-Generation BioGreen 21 Program of the Rural Development Administration, Republic of Korea [SSAC means System & Synthetic Agro-biotech Center, grant PJ01116602].

References


