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Lysobacter fragariae sp. nov. and *Lysobacter rhizosphaerae* sp. nov. isolated from rhizosphere of strawberry plant

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Abstract Two bacterial strains, designated THG-DN8.7^T and THG-DN8.3^T, were isolated from the rhizosphere of a strawberry plant in Gyeryong Mountain, South Korea. Cells of both isolates were observed to be Gram-negative, yellow-coloured and rod-shaped. Comparative 16S rRNA gene sequence analysis showed that strain THG-DN8.7^T had highest sequence similarities to *Lysobacter yangpyeongensis* KACC 11407^T (97.2 %), *Lysobacter niabensis* KACC 11587^T (97.0 %) and *Lysobacter oryzae* KCTC 22249^T (96.9 %), while strain THG-DN8.3^T had closely similarity with *L. niabensis* KACC 11587^T (98.1 %), *L. oryzae* KCTC 22249^T (97.1 %) and *L. yangpyeongensis* KACC 11407^T (96.1 %). DNA–DNA relatedness values

between strains THG-DN8.7^T and THG-DN8.3^T and their closest phylogenetically neighbours were below 30.0 %, which indicates that strains THG-DN8.7^T and THG-DN8.3^T represent distinct species within the genus *Lysobacter*. Both strains were found to contain iso-C_{15:0}, iso-C_{16:0} and iso-C_{17:1}ω9c as predominant fatty acids and ubiquinone-8 as major isoprenoid quinone. The major polar lipids were identified as phosphatidylethanolamine, phosphatidyl-N-methylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content of strains THG-DN8.7^T and THG-DN8.3^T were determined to be 66.9 and 67.8 mol%, respectively. These data are consistent with the affiliation of the two new species represented by THG-DN8.7^T and THG-DN8.3^T to the genus *Lysobacter*. The names *Lysobacter fragariae* sp. nov. and *Lysobacter rhizosphaerae* sp. nov. are proposed for these species with the type strains THG-DN8.7^T (=KCTC 42236^T = JCM 30322^T) and THG-DN8.3^T (=KCTC 42237^T = JCM 30321^T), respectively.

Hina Singh and Juan Du have equally contributed to this work.

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Keywords *Lysobacter fragariae* · *Lysobacter rhizosphaerae* · Gram-negative · 16S rRNA · Ubiquinone-8

Introduction

The genus *Lysobacter* was proposed by Christensen and Cook (1978) with *Lysobacter enzymogenes* as the

type species. Members of the genus *Lysobacter* are rod-shaped, lack flagella, have high G+C content (61.7–70.7 %) and contain ubiquinone-8 (Q-8) as their predominant isoprenoid quinone (Lee et al. 2006; Wei et al. 2012). Their major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidyl-*N*-methyl-ethanolamine (Park et al. 2008). At the time of writing, following the reclassification of *Lysobacter thermophilus* (Yu et al. 2013), the genus *Lysobacter* contains 26 species with validly published names (<http://www.bacterio.net/lysobacter.html>) and several new species have been recently been described, including *Lysobacter terrae* (Ngo et al. 2014), *Lysobacter mobilis* (Yang et al. 2014), *Lysobacter caeni* (Ye et al. 2014) and *Lysobacter lycopersici* (Lin et al. 2015). In this study, the taxonomic position of two strains isolated from the rhizosphere of a strawberry plant, THG-DN8.7^T and THG-DN8.3^T were investigated by means of a polyphasic approach.

Materials and methods

Isolation of bacterial strains

A soil sample was collected from the rhizosphere of a strawberry plant (*Fragaria vesca* L.) in Gyeryong Mountain, South Korea. Strains THG-DN8.7^T and THG-DN8.3^T were isolated by a conventional dilution-plating method using Reasoner's 2A agar (R2A agar; Difco). The isolates were routinely cultured on R2A agar at 28 °C and stored in nutrient broth containing glycerol suspension (25 %, w/v) at –80 °C. For comparative study, the reference type strains of *Lysobacter yangpyeongensis* KACC 11407^T, *Lysobacter niabensis* KACC 11587^T, *Lysobacter oryzae* KCTC 22249^T, *Lysobacter terrae* KACC 17646^T and *Lysobacter enzymogenes* KACC 10127^T were cultured under the same conditions as strains THG-DN8.7^T and THG-DN8.3^T.

Molecular characterization and phylogenetic construction

Genomic DNA of strains THG-DN8.7^T and THG-DN8.3^T were extracted and purified using a Solgent genomic DNA extraction kit (Korea). The 16S rRNA genes were amplified with the universal bacterial

primer pair 27F (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Weisburg et al. 1991). The 16S rRNA gene sequencing was performed by Solgent Co. Ltd (Korea). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and EzTaxon e-server (Kim et al. 2012). The multiple alignments were performed using the CLUSTAL_X program (Thompson et al. 1997) followed by gap editions in the BioEdit program (Hall 1999). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura 1983). The phylogenetic trees were constructed using the neighbour-joining (Saitou and Nei 1987) and maximum-likelihood methods in the MEGA 4 and MEGA 5 program, respectively (Tamura et al. 2011). The bootstrap values were calculated based on 1000 replications (Felsenstein 1985).

For determination of the DNA G+C content, genomic DNA of strains THG-DN8.7^T and THG-DN8.3^T were prepared (Moore and Dowhan 1995) and degraded enzymatically into nucleosides (nuclease P1 and alkaline phosphatase; Sigma). The G+C contents were analyzed using a reverse-phase HPLC system (Alliance 2690 system, Waters) as described by Mesbah et al. (1989).

DNA–DNA hybridizations were performed fluorometrically, according to the method developed by Ezaki et al. (1989) with modifications (Stabili et al. 2008), using photobiotin-labelled DNA probes and micro-dilution wells. DNA–DNA hybridization experiments were performed between strains THG-DN8.7^T and THG-DN8.3^T and their closely related strains, with the hybridization temperature at 45 °C. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage DNA–DNA relatedness values.

Chemotaxonomic characterization

For cellular fatty acid analysis, strains THG-DN8.7^T, THG-DN8.3^T and the reference strains were harvested from R2A plates after incubation for 2 days at 28 °C. The fatty acids were prepared according to the protocol of Sherlock microbial identification system (MIDI) and identified with GC (Hewlett Packard 6890) using the Sherlock Aerobic Bacterial Database (TSBA60) (Sasser 1990).

For quinone and polar lipid analyses, cells were cultured in R2A broth for 2 days and freeze-dried after harvesting. Respiratory quinones were extracted and subsequently analyzed using a Waters RP-HPLC system (Alliance 2690 system) as previously described (Hiraishi et al. 1996; Collins and Jones, 1981; Tamaoka et al. 1983). Polar lipids of strain THG-DN8.7^T, THG-DN8.3^T and their most closely related reference strains *L. yangpyeongensis* KACC 11407^T and *L. niabensis* KACC 11587^T were analyzed using 2-dimensional thin-layer chromatography (Minnikin et al. 1984; Tindall 1990). For the detection of total and specific lipids, the following reagents were used: 5 % molybdatophosphoric acid (total lipids, Sigma); 0.2 % ninhydrin (aminolipids, Sigma); 2.5 % α -naphthol-sulfuric acid (glycolipids, Sigma); and molybdenum blue (phospholipids, Sigma).

Morphological and physiological characterization

Gram reaction was determined using a bioMérieux (France) Gram stain kit according to the manufacturer's instructions. Cell morphology of strains THG-DN8.7^T and THG-DN8.3^T was observed by transmission electron microscope (Model JEM1010; JEOL) at $\times 11,000$ magnification, using cells grown for 2 days at 28 °C on R2A agar. Growth at different temperatures (4, 10, 15, 18, 25, 28, 30, 35, 37 and 42 °C) was tested on R2A agar. Growth at pH conditions (pH 4.0–10.0, at intervals of 0.5 pH units) and salt tolerance [0–5.0 % NaCl (w/v), at intervals of 0.5 %] were assessed in R2A broth. Motility testing was performed by the hanging-drop technique using cells grown for 2 days at 28 °C in R2A broth (Skerman 1967) and sulfide-indole-motility medium (SIM; Difco). Anaerobic growth was tested in serum bottles containing R2A broth supplemented with thioglycolate (0.1 %), in which the air was substituted with nitrogen gas. Oxidase and catalase activity were tested by 1 % (w/v) *N*, *N*, *N'*, *N'*-tetramethyl-1,4-phenylenediamine reagent and 3 % (v/v) H₂O₂, respectively. Tests for hydrolysis were performed on R2A agar containing (w/v): casein (2 % skim milk, Oxoid); starch (1 %, Difco); Tween 80 (0.01 % CaCl₂•2H₂O and 1 % Tween 80, Sigma); Tween 20 (0.01 % CaCl₂•2H₂O and 1 % Tween 20, Sigma); chitin (1 %, Sigma); L-tyrosine (0.5 %, Sigma); carboxymethyl-cellulose (CMC) [0.1 %, Sigma]; DNA (DNase agar, Oxoid) and aesculin (Bile Esculin agar,

Difco). Growth on nutrient agar (NA, Oxoid), tryptone soya agar (TSA, Oxoid), Luria–Bertani agar (LB agar; Oxoid), marine agar (MA, Oxoid) and MacConkey agar (Oxoid) were also tested. All the above tests, unless specifically indicated, were evaluated after 5 days incubation at 28 °C. In addition, tests of carbon-source utilisation and enzyme activities were performed with API 20NE and API ZYM kits according to the instructions of the manufacturer (bioMérieux, France).

Results and discussion

Two bacterial strains, designated THG-DN8.7^T and THG-DN8.3^T, were isolated from the rhizosphere of a strawberry plant. The 16S rRNA gene sequence similarity between strains THG-DN8.7^T (1,447 bp) and THG-DN8.3^T (1,458 bp) is 98.0 %. According to the EzTaxon server, sequence similarities indicated that strain THG-DN8.7^T shows the highest similarity to *L. yangpyeongensis* KACC 11407^T (97.2 %), followed by *L. niabensis* KACC 11587^T (97.0 %) and *L. oryzae* KCTC 22249^T (96.9 %), while strain THG-DN8.3^T has closely similarity with *L. niabensis* KACC 11587^T (98.1 %), *L. oryzae* KCTC 22249^T (97.1 %) and *L. yangpyeongensis* KACC 11407^T (96.1 %). In addition, the phylogenetic trees (Fig. 1 and Supplementary Fig. S1) indicated that strains THG-DN8.7^T and THG-DN8.3^T have a close phylogenetic relationship with *L. terrae* THG-A13^T (KF483861), which shows 98 % 16S rRNA gene sequence identities using the NCBI BLAST server. Strain THG-DN8.7^T showed a low DNA–DNA relatedness to THG-DN8.3^T (25.5 \pm 0.5 %), *L. terrae* KACC 17646^T (20.3 \pm 1.0 %), *L. yangpyeongensis* KACC 11407^T (29.5 \pm 0.5 %) and *L. niabensis* KACC 11587^T (23.4 \pm 1.5 %). The level of DNA–DNA relatedness for strain THG-DN8.3^T with respect to THG-DN8.7^T, *L. terrae* KACC 17646^T, *L. niabensis* KACC 11587^T and *L. oryzae* KCTC 22249^T were 23.5 \pm 0.5, 22.9 \pm 1.0, 28.5 \pm 0.5 and 4.1 \pm 1.5 %, respectively. These low levels of DNA–DNA relatedness suggest that strains THG-DN8.7^T and THG-DN8.3^T are novel *Lysobacter* species (Stackebrandt and Goebel 1994) and genotypically distant from each other.

Both strains were found to contain iso-C_{15:0}, iso-C_{16:0} and iso-C_{17:1} ω 9c as predominant fatty acids

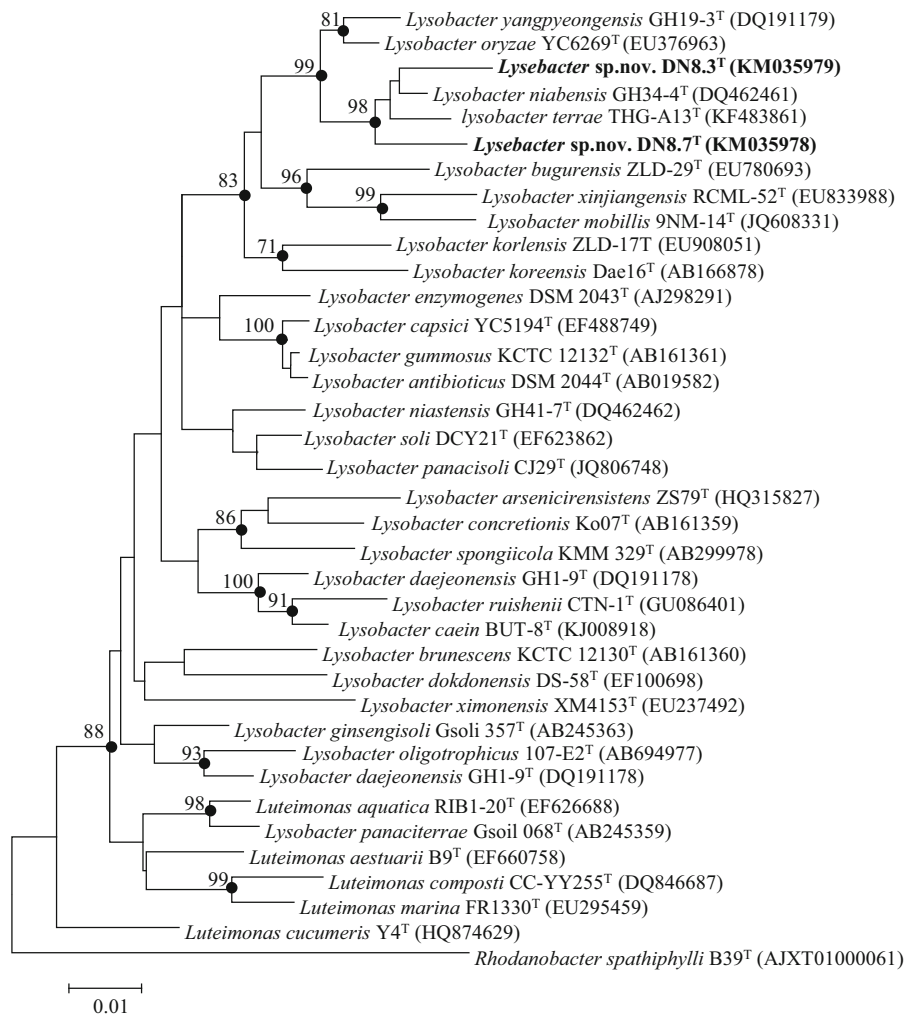


Fig. 1 A neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the relationships of strains THG-DN8.7^T, THG-DN8.3^T and members of the genus *Lysobacter*. Filled circles indicate that the bootstrap values were

more than 70 % based on 1000 replications. *Rhodanobacter spathiphylli* B39^T (AJXT01000061) was used as outgroup. Scale bar 0.01 substitutions per nucleotide position

(Table 1) and ubiquinone-8 as their major isoprenoid quinone. The major polar lipids were identified as phosphatidylethanolamine, phosphatidyl-*N*-methylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (Supplementary Fig. 2). These data are consistent with the affiliation of the two new species represented by THG-DN8.7^T and THG-DN8.3^T to the genus *Lysobacter*. The DNA G+C content of strains THG-DN8.7^T and THG-DN8.3^T were determined to be 66.9 and 67.8 mol%, respectively, which is in the range for the members of the genus *Lysobacter*.

Morphological observation showed strain THG-DN8.7^T colonies on R2A agar to be yellow, round, sticky and raised with approximate diameter 3–5 mm while those of strain THG-DN8.3^T colonies on R2A agar were observed to be pale yellow, smooth, convex with entire margins and approximate diameter 1–3 mm after incubation at 28 °C for 48 h. Both strains THG-DN8.7^T and THG-DN8.3^T were found to grow well on R2A agar and NA, to grow weakly on TSA and LB agar, but not to grow on MA and MacConkey agar. Strain THG-DN8.7^T grows optimally in R2A at 10–28 °C (optimum 25–28 °C), at

Table 1 Cellular fatty acid composition of strains THG-DN8.7^T, THG-DN8.3^T and the type strains of closely related species

Characteristic	1	2	3	4	5	6	7
Oxidase	+	–	+	+	+	+	+
Motility	–	–	–	+	+	–	+
Nitrate reduction	–	–	–	+	–	–	–
<i>Hydrolysis of</i>							
Starch	–	+	–	+	–	+	+
Casein	+	+	+	+	–	+	+
CMC	+	–	+	+	+	+	+
Tween 20	–	+	+	–	+	–	–
Tween 80	–	–	+	+	+	+	+
Gelatin	+	+	W	+	W	W	+
L-tyrosine	+	+	+	+	+	+	+
DNA	–	+	–	+	+	–	–
Esculin	–	–	+	–	+	–	+
<i>Assimilation of</i>							
β -galactosidase	–	–	+	W	W	+	+
N-acetyl-glucosamine	–	–	–	–	–	–	+
D-maltose	–	–	–	–	–	–	+
Trisodium citrate	–	–	–	–	–	+	–
Malic acid	–	–	–	–	–	–	+
Capric acid	–	–	+	–	–	–	+
<i>Enzyme activity</i>							
Lipase (C14)	–	–	W	+	+	W	+
Valine arylamidase	W	+	+	+	+	+	+
Cysteine arylamidase	–	+	+	+	+	+	+
Trypsin	–	+	+	+	W	+	+
α -chymotrypsin	–	W	+	+	W	+	+
Naphtol-AS-BI-phosphohydrolase	W	+	+	+	+	+	+
α -glucosidase	–	–	W	+	+	+	+
β -glucosidase	–	–	+	+	+	+	+
N-acetyl- β -glucosaminidase	–	+	+	+	–	–	+

Strains 1 THG-DN8.7^T; 2 THG-DN8.3^T; 3 *L. terrae* KACC 17646^T; 4 *L. yangpyeongensis* KACC 11407^T; 5 *L. niabensis* KACC 11587^T; 6 *L. oryzae* KCTC 22249^T; 7 *L. enzymogenes* ATCC 29487^T (type strain of the genus). All the data were obtained in this study
(+) Positive, (W) weakly positive, (–) negative

pH 6.5–8.5 (optimum 7.0–7.5) and in presence of 0–1.0 % NaCl while strain THG-DN8.3^T grows optimally in R2A at 18–28 °C (optimum 25–28 °C), at pH 6.0–8.0 (optimum 6.5–7.5) and in presence of 0–0.5 % NaCl. Phenotypic analysis showed that strain THG-DN8.7^T cells are Gram-negative, non-motile, aerobic and rod-shaped with size range approximately 0.7–0.9 \times 3.4–4.7 μ m (Supplementary Fig. S3). The tests for oxidase and catalase activities were positive. Nitrate reduction and indole production was found to be negative. Strain THG-DN8.7^T was not able to hydrolyse starch, Tween 80, Tween 20, aesculin, chitin and DNA but is able to hydrolyse L-tyrosine, casein and CMC. Cells of strain THG-DN8.3^T are Gram-negative,

facultative anaerobic, non-motile and rod-shaped (0.5–0.9 \times 3.0–4.0 μ m) (Supplementary Fig. S3). The test for catalase was found to be positive. Oxidase test, nitrate reduction and indole production were found to be negative. Strain THG-DN8.3^T is able to hydrolyse starch, L-tyrosine, Tween 20, DNA and casein but unable to hydrolyse Tween 80, CMC, aesculin and chitin. The biochemical and physiological characteristics of strains THG-DN8.7^T, THG-DN8.3^T and the most closely related *Lysobacter* type strains are given in Table 2. The results suggest that the two novel isolates represent novel species of the genus *Lysobacter*.

On the basis of the phylogenetic, phenotypic and chemotaxonomic data, strains THG-DN8.7^T (=KCTC

Table 2 The differential biochemical and physiological characteristics of strains THG-DN8.7^T and THG-DN8.3^T and closely related type strains of species of genus *Lysobacter*

Fatty acid	1	2	3	4	5	6	7
Saturated							
C _{16:0}	8.5	5.9	6.0	9.1	11.7	6.9	9.8
Unsaturated							
C _{16:1} ω7c alcohol	8.0	4.7	7.7	7.9	Tr	3.6	Tr
iso-C _{17:1} ω9c	16.0	12.0	11.0	5.7	5.1	16.5	8.3
Branched-chain							
iso-C _{11:0}	3.9	3.4	4.7	3.2	1.3	5.2	3.3
iso-C _{11:0} 3OH	5.6	4.6	7.7	5.2	1.8	6.5	6.6
iso-C _{14:0}	1.6	5.8	3.2	4.1	1.4	1.7	Tr
iso-C _{15:0}	12.4	11.4	18.6	12.9	6.4	17.7	20.5
anteiso-C _{15:0}	Tr	2.5	5.9	4.2	5.9	2.9	3.5
iso-C _{16:0}	22.4	31.2	16.4	23.5	6.9	17.3	14.0
iso-C _{17:0}	5.7	1.7	3.8	1.8	2.1	9.9	1.8

Strains 1 THG-DN8.7^T; 2 THG-DN8.3^T; 3 *L. terrae* KACC 17646^T; 4 *L. yangpyeongensis* KACC 11407^T; 5 *L. niabensis* KACC 11587^T; 6 *L. oryzae* KCTC 22249^T; 7 *L. enzymogenes* ATCC 29487^T. All the data were obtained in this study, cells were cultured on R2A agar for 2 days at 28 °C. Tr traces (<1.0 %)

42236^T = JCM 30322^T) and THG-DN8.3^T (=KCTC 42237^T = JCM 30321^T) are considered to represent novel species of the genus *Lysobacter*, for which names *Lysobacter fragariae* sp. nov. and *Lysobacter rhizosphaerae* sp. nov. are proposed.

Description of *Lysobacter fragariae* sp. nov

Lysobacter fragariae (fra.ga'ri.ae. N.L. gen. n. *fragariae* of a strawberry).

Cells are Gram-negative, aerobic, non-motile and rod-shaped (0.7–0.9 × 3.4–4.7 μm). Grows well on R2A agar and NA, grows weakly on TSA and LB agar, but does not grow on MA and MacConkey agar. Growth occurs at 10–28 °C (optimum 25–28 °C), at pH 6.5–8.5 (optimum 7.0–7.5) and at 0–1.0 % NaCl. Catalase and oxidase activities are positive. L-Tyrosine, casein and CMC are hydrolysed but starch, Tween 80, Tween 20, aesculin, chitin and DNA are not. In API 20NE tests, nitrate reduction, indole production, glucose acidification, arginine dihydrolase, β-galactosidase, β-glucosidase and urease are negative and gelatin hydrolysis (protease) is positive. The following compounds are not utilised as a sole source of carbon: D-glucose, L-arabinose, D-mannose, D-mannitol,

N-acetyl-glucosamine, D-maltose, potassium gluconate, adipic acid, malic acid, trisodium citrate, phenylacetic acid and capric acid. In API ZYM tests, positive results are obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and acid phosphatase; weakly positive results are obtained for valine arylamidase and naphthol-AS-BI-phosphohydrolase; negative results are obtained for lipase (C14), cysteine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-fucosidase, α-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, β-galactosidase, β-glucosidase and α-mannosidase. Characteristics used for differentiation from other *Lysobacter* species are given in Table 2. The major polar lipids are phosphatidylethanolamine, phosphatidyl-N-methylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The predominant isoprenoid quinone is Q-8. The major fatty acids are iso-C_{15:0}, iso-C_{16:0} and iso-C_{17:1} ω9c. The DNA G+C content of the type strain is 66.9 mol%.

The type strain, THG-DN8.7^T (=KCTC 42236^T = JCM 30322^T), was isolated from the rhizosphere of a strawberry plant in Gyeryong Mountain, South Korea. The NCBI GenBank accession number for the 16S rRNA gene sequence of strain THG-DN8.7^T is KM035978.

Description of *Lysobacter rhizosphaerae* sp. nov

Lysobacter rhizosphaerae [rhi.zo.sphae'rae. Gr. fem. n. *rhiza* root; L. fem. n. *sphaera* -ae (from Gr. fem. n. *sphaira* -as) ball, any globe, sphere; N.L. gen. fem. n. *rhizosphaerae* of the rhizosphere].

Cells are Gram-negative, facultative anaerobic, non-motile, catalase positive, oxidase negative and rod-shaped ($0.5\text{--}0.9 \times 3.0\text{--}4.0 \mu\text{m}$). Grows well on R2A agar and NA, grows weakly on TSA and LB agar, but does not grow on MA and MacConkey agar. Growth occurs at $18\text{--}28^\circ\text{C}$ (optimum $25\text{--}28^\circ\text{C}$), at pH $6.0\text{--}8.0$ (optimum $6.5\text{--}7.5$) and at $0\text{--}0.5\%$ NaCl. Starch, L-tyrosine, Tween 20, DNA and casein are hydrolysed but Tween 80, CMC, aesculin and chitin are not. In API 20NE tests, nitrate reduction, indole production, glucose acidification, arginine dihydrolase, β -galactosidase, β -glucosidase and urease are negative and gelatin hydrolysis (protease) is positive. The following compounds are not utilised as a sole source of carbon: D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, adipic acid, malic acid, trisodium citrate, phenylacetic acid and capric acid. In API ZYM tests, positive results are obtained for alkaline phosphatase, esterase (C4), valine arylamidase, esterase lipase (C8), leucine arylamidase, cysteine arylamidase, trypsin, *N*-acetyl- β -glucosaminidase, naphthol-AS-BI-phosphohydrolase and acid phosphatase; weakly positive result is obtained for α -chymotrypsin; negative results are obtained for lipase (C14), β -glucuronidase, α -fucosidase, α -galactosidase, α -glucosidase, β -galactosidase, β -glucosidase and α -mannosidase. Characteristics used for differentiation from other *Lysobacter* species are given in Table 2. The major isoprenoid quinone is Q-8. The major polar lipids are phosphatidylethanolamine, phosphatidyl-*N*-methylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The major fatty acids are iso $\text{C}_{15:0}$, iso $\text{C}_{16:0}$ and iso $\text{C}_{17:1\omega 9c}$. The DNA G+C content of the type strain is 67.8 mol%.

The type strain, THG-DN8.3^T (=KCTC 42237^T = JCM 30321^T), was isolated from the rhizosphere of a strawberry plant in Gyeryong Mountain, South Korea. The NCBI GenBank accession number for the 16S rRNA gene sequence of strain THG-DN8.3^T is KM035979.

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