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ORIGINAL PAPER

Lysobacter agri sp. nov., a bacterium isolated from soil

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Abstract A bacterial strain, designated as THG-SKA3^T, was isolated from field soil of Kyung Hee University, South Korea. Cells of the isolate were observed to be Gram-negative, aerobic, rod-shaped and motile by gliding. The strain was found to grow optimally at 28 °C, at pH 7 and in absence of NaCl. Based on 16S rRNA gene sequence comparisons, strain THG-SKA3^T shared highest sequence similarity with *Lysobacter niastensis* KACC 11588^T followed by *Lysobacter panacisoli* KACC 17502^T, *Lysobacter enzymogenes* LMG 8762^T and *Lysobacter oryzae* KCTC 22249^T. The G+C content of THG-SKA3^T was determined to be 68.9 mol%. The DNA–DNA relatedness values between strain THG-SKA3^T and its closest phylogenetic neighbors were below 25.0 %.The major polar lipids of strain THG-SKA3^T were determined to be diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The predominant respiratory quinone was identified as ubiquinone 8 (Q-8). The major cellular fatty acids were identified as branched chain iso-$C_{15:0}$, iso-$C_{16:0}$ and unsaturated iso-$C_{17:1}\omega 9c$. On the basis of polyphasic data presented, it is evident that strain THG-SKA3^T represents a novel species of the genus *Lysobacter*, for which the name *Lysobacter agri* sp. nov. (type strain THG-SKA3^T = KACC 18283^T = CSCTCC AB 2015126^T) is proposed.

Keywords *Lysobacter agri* · Polyphasic taxonomy · Gram-negative · Ubiquinone Q-8

Introduction

The genus *Lysobacter* was first proposed by Christensen and Cook (1978), with *Lysobacter enzymogenes* as the type species. The genus *Lysobacter* is classified in the family *Xanthomonadaceae*. Members of the genus are generally characterized as Gram-negative, aerobic, non-fruiting and gliding bacteria with a high DNA G+C content typically ranging from 61.7 to 70.1 mol% (Christensen and Cook 1978; Weon et al. 2006). Species of the genus *Lysobacter* contain ubiquinone 8 (Q-8) as the major respiratory

The NCBI GenBank accession number for the 16S rRNA gene sequence of strains THG-SKA3^T is KM576858.

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quinone and a predominance of iso-branched fatty acids (Saddler and Bradbury 2005; Luo et al. 2012; Wei et al. 2012; Fukuda et al. 2013). Predominance of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine is a typical characteristic of the genus (Park et al. 2008; Romanenko et al. 2008; Wang et al. 2009; Zhang et al. 2011). All described species of *Lysobacter* showed negative results for urease activity and indole production (Ten et al. 2009; Zhang et al. 2011). *Lysobacter* species are typically found in soil and water habitats (Ten et al. 2009; Srinivasan et al. 2010; Liu et al. 2011). 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). Many species have been reported recently such as *Lysobacter terrae* (Ngo et al. 2014), *Lysobacter mobilis* (Yang et al. 2014), *Lysobacter caeni* (Ye et al. 2014), *Lysobacter lycopersici* (Lin et al. 2015), *Lysobacter fragariae* and *Lysobacter rhizosphaerae* (Singh et al. 2015). The species of the genus *Lysobacter* are related to members of the genera *Xanthomonas*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Thermomonas, Vulcaniibacterium* and *Xylella*.

In this study, we characterized a new isolate belonging to the genus *Lysobacter* isolated from field soil of Kyung Hee University, South Korea. The phenotypic and genotypic characterizations of the novel strain are described in this report.

Materials and methods

Isolation and phylogenetic analysis

A soil sample was collected from field of Kyung Hee University, South Korea. One gram of sample was suspended in 10 ml of 0.85 % (w/v) saline solution, vortexed, serially diluted up to 10^{-5} and spread on Reasoner's 2A agar (R2A; Difco, USA). The plates were incubated at 28 °C for one week and strain THG-SKA3^T was subsequently isolated. Firstly, the isolates were routinely cultured on R2A agar at 28 °C and preserved as suspension in R2A broth with 25 % (v/v) glycerol and stored at −80 °C. Strain THG-SKA3^T has been deposited in the Korean Agricultural Culture Collection (KACC 18283^T) and China Centre for Type Culture Collection (CCTCC AB 2015126^T). The reference strains (Table 1) were obtained from various culture collection centers. These strains were cultured under the same optimum conditions as strain THG-SKA3^T.

Genomic DNA was extracted and purified using a commercial Genomic DNA extraction kit (Solgent, Korea). The 16S rRNA genes were amplified using universal bacterial primer pair 27F and 1492R (Weisburg et al. 1991) and the purified PCR products were sequenced by Solgent Co. Ltd (Daejeon, Korea). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and EzTaxon e–server [http://eztaxon-e.ezbiocloud.net/; Kim et al. (2012)]. Full sequences of the 16S rRNA gene were compiled using Seq-Man software version 4.1 (DNASTAR Inc, USA). The multiple alignments were performed using the CLUSTAL_X program (Thompson et al. 1997) and gaps were edited using 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 neighbor-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971) and maximum-likelihood methods (Felsenstein 1981) in the MEGA 6 program package (Tamura et al. 2013) with bootstrap values based on 1000 replications (Felsenstein 1985).

Phenotypic analysis

Gram staining was performed using a Gram stain kit according to the manufacturer's instructions (bioMérieux, France). Cells were grown in R2A broth for 24 h at 28 °C and then tested for gliding motility by the hanging-drop technique (Skerman 1967). For transmission electron microscopy suspended cells previously grown on R2A agar at 28 °C for 48 h were placed on carbon and Formvar-coated nickel grids for 30 s. Grids were floated on 1 drop of 0.1 % (w/v) aqueous uranyl acetate, blotted dry and then viewed with a (Model JEM1010; JEOL) at ×11,000 magnification under standard operating conditions. Growth at different temperature (4, 10, 15, 18, 25, 28, 30, 35, 37 and 42 °C) was tested on R2A agar for 7 days. Different media were tested for growth such as nutrient (NA, Difco), trypticase soy (TSA; Difco), R2A agar and MacConkey (Difco), luria–bertani (LB, Difco) and marine (MB; Difco) agars at 28 °C for 7 days. Tolerance for salinity was evaluated in R2A broth supplemented with [0–5.0 % (w/v) NaCl, at

Table 1 The different biochemical, physiological characteristics of strain THG-SKA3^T and related type strains

Characteristics	1	2	3	4	5
Colony color	W	LB	BY	DY	PY
Nitrate reduction	+	+	−	−	−
Gliding Motility	+	+	−	+	+
Oxidase	+	+	−	+	+
Flexirubin	−	+	+	−	−
Hydrolysis of					
Tyrosine	+	+	−	+	+
DNA	−	−	+	+	−
Tween 80	−	+	−	+	+
Starch	+	+	−	+	+
CMC	+	−	−	+	+
Chitin	−	−	−	+	−
Gelatin	−	+	+	+	+
Esculin	+	−	+	+	+
Utilization of carbon source					
Arginine dihydrolase	−	−	−	+	−
D-Glucose	+	−	−	+	−
L-Arabinose	+	−	−	−	−
D-Mannose	+	−	+	+	−
D-Mannitol	+	−	−	−	−
N-acetyl-glucosamine	+	+	−	−	−
D-Maltose	+	+	−	+	−
Gluconate	+	−	−	−	−
Caprate	−	−	−	−	+
Malate	−	−	−	+	−
Citrate	−	−	−	+	−
Enzyme activity					
Lipase (C14)	+	+	−	+	−
Cystine arylamidase	+	−	+	+	−
Trypsin	−	+	+	+	+
α-Chymotrypsin	−	+	+	+	+
α-Galactosidase	+	−	−	−	−
β-Galactosidase	+	+	−	−	−
β-Glucosidase	+	+	+	−	−
N-acetyl-β-glucosaminidase	+	−	+	+	−
α-Mannosidase	+	−	−	−	−
DNA G+C content (mol %)	68.9	66.6[a]	65.6[b]	69.0[c]	67.4[d]

All strains were positive for catalase and hydrolysis of casein. All strains were negative for following characteristics: hydrolysis of Tween 20 and urea; indole production; glucose acidification; for assimilation of adipate and phenyl acetate. In API ZYM strips, all strains were positive for alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, α-glucosidase and Naphthol-*AS-BI*-phosphohydrolase but negative for β-glucuronidase and α-fucosidase. All data were obtained from this study except the DNA G+C content of reference strains: [a] data from Weon et al. (2007), [b] data from Choi et al. (2014), [c] and [d] data from Aslam et al. (2009)

Strains: *1* THG-SKA3^T; *2 L. niastensis* KACC 11588^T; *3 L. panacisoli* KACC 17502^T; *3 L. enzymogenes* LMG 8762^T; *4 L. oryzae* KCTC 22249^T

W white, *LB* light Beige, *BY* bright yellow, *DY* dark yellow, *PY* pale yellow, + Positive, − negative

0.5 % intervals]. The pH range for growth was examined at pH 4.0–10.0 by 0.5 pH unit intervals in R_2A broth adjusted with 10 mM phosphate-citrate buffer (pH 4.0–5.0), MES buffer (pH 5.5–6.5), HEPES buffer 91 (pH 7.0–8.0), Tris buffer (pH 8.5–9.0) and $NaHCO_3/Na_2CO_3$ (pH 9.5–10.0). The pH of R2A broth was confirmed after autoclaving. The growth was estimated by monitoring the optical density at 600 nm after 5 days of incubation at 28 °C. Catalase activity was determined by the formation of bubbles in 3 % (v/v) H_2O_2 solution. Oxidase activity was determined by using 1 % (w/v) *N,N,N,N*,-tetramethyl-1,4-phenylenediamine reagent (Sigma, USA) according to the manufacturer's instructions. Anaerobic growth was tested in serum bottles containing R2A broth supplemented with thioglycolate (0.1 %) and in which the air was substituted with nitrogen gas. The presence of flexirubin-type pigments was investigated as described by Reichenbach (1992), Schmidt et al. (1994) and Bernardet et al. (2002). Hydrolysis of the following substrates was tested using R2A as basal medium: 2 % Caesin (skim milk, Oxoid, England), 1 % starch (Difco), 0.1 % esculin (0.02 % ferric citrate, Difco), 12 % gelatin (Sigma), Tween 80 [0.01 % $CaCl_2{\cdot}2H_2O$ and 1 % Tween 80 (Sigma)], Tween 20 [0.01 % $CaCl_2{\cdot}2H_2O$ and 1 % Tween 20 (Sigma)], 1 % chitin (Sigma), 0.5 % L-tyrosine (Sigma), 0.1 % carboxymethyl-cellulose (CMC, Sigma) and DNA [DNase agar, Scharlau (Spain); DNase activity revealed by flooding the plates with 1 N HCl]. Plates were evaluated after 5 days of incubation at 28 °C. Indole production was analyzed using Kovacs' reagent in 1 % tryptone broth. Nitrate reduction was tested in nitrate broth containing 0.2 % KNO_3 (Skerman 1967). Urease activity was evaluated in Christensen's medium (Christensen 1946). Carbon-source assimilation and enzyme activity were assessed using API 20NE and API ZYM kits at 28 °C according to the manufacturer's protocols (bioMérieux, France). The results of API 20NE strips were recorded after incubation for 48 h and API ZYM strips were recorded after 10 h of incubation.

Determination of DNA G+C content

Genomic DNA of strain THG-SKA3^T was extracted and purified by the method described by Moore and Dowhan (1995). Nuclease and alkaline phosphatase enzymes were used to degrade DNA nucleotides into single nucleosides. The obtained nucleosides was separated using a reverse-phase HPLC system (Alliance 2690 system, Waters) as described previously by (Mesbah et al. 1989) with reversed-phase column SunFireTM C18 (4.6 × 250 mm × 5 µm), flow rate of 1.0 ml/min, solvent mixture of 200 mM $(NH_4)H_2PO_4$/acetonitrile (97:3, v/v) as mobile phase and detector wavelength at 270 nm. The genomic DNA of *Escherichia coli* strain B (Sigma-Aldrich D4889) was used as a standard.

DNA–DNA hybridization

DNA–DNA hybridization was 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 was carried out to determine levels of relatedness of the novel strain THG-SKA3^T with its closest relatives *Lysobacter niastensis* KACC 11588^T and *Lysobacter panacisoli* KACC 17502^T. The optimum renaturation temperature (46 °C) was calculated as [(0.51 × G+C content) + 47] − 36 (Gillis et al. 1970), where 36 °C is the correction for the presence of 50 % formamide (McConaughy et al. 1969). 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.

Cellular fatty acids, Quinone and polar lipid composition

For fatty acid analysis, all strains were grown on R2A agar at 28 °C for 48 h. Cells were then harvested. Fatty acid were extracted, methylated and saponified by the method described by Sherlock Microbial Identification system (MIDI) and analyzed by capillary GLC (Hewlet Packard 6890) using the TSBA library version 6.1 (Sasser 1990). For quinone and polar lipids analysis freeze dried cells of strains THG-SKA3^T and *L. niastensis* KACC 11588^T were used. Ubiquinone was extracted from freeze dry cell (300 mg) with chloroform: methanol (2:1, v/v) afterward concentrated at 40 °C using vacuum rotary evaporator, the residue was subsequently extracted with 10 ml hexane. After purification by Sep-PakR

Vac 6 cc silica cartridge, the samples were analyzed by using a reverse-phase HPLC system (Alliance 2690 system, Waters) [wavelength 270 nm, solvent MeOH: isopropanol (7:5, v/v), flow rate; 1.0 ml/min] as previously described (Hiraishi et al. 1996; Collins and Jones 1981; Tamaoka et al. 1983). The polar lipids of strain THG-SKA3^T and its closest reference strain *L. niastensis* KACC 11588^T were analyzed as described by Minnikin et al. (1984). Two-dimensional thin layer chromatography (2D-TLC) performed using TLC Kiesel gel 60 F_{254} plates (10 × 10 cm; Merck, USA). Separately, each sample was spotted on the corner of and the plates were first developed with chloroform: methanol: water (65:25:4, v/v/v) followed by a second development with chloroform: methanol:acetic acid:water (80:12:15:4, v/v/v/v). TLC plates were sprayed with following reagents: 5 % molybdophosphoric acid (total lipids, Sigma), 0.2 % ninhydrin (aminolipids, Sigma) and 15 % α-naphthol-sulfuric acid (glycolipids, Sigma). After spraying, it followed by heating at 120 °C for 10 min. TLC plates also sprayed with Molybdenum blue reagent for detecting phospholipids. No heating step needed for this reagent.

Results and discussion

Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain THG-SKA3^T formed a monophyletic cluster with the members of the genus *Lysobacter* (Fig. 1). This cluster was also recovered in the tree generated by the maximum-parsimony algorithm. Highest sequence similarity was observed with *Lysobacter niastensis* KACC 11588^T (96.9 %) followed by *Lysobacter panacisoli* KACC 17502^T (96.9 %), *Lysobacter enzymogenes* LMG 8762^T (96.2 %) and *Lysobacter oryzae* KCTC 22249^T (96.1 %). Strain THG-SKA3^T also shows less than 95 % similarity with other members of the family *Sphingobacteriaceae*. A phylogenetic tree built using the maximum-likelihood method is presented as Supplementary Fig. S1. The phylogenetic data indicated that strain THG-SKA3^T represents a novel species of the genus *Lysobacter*. The DNA G+C content of strain THG-SKA3^T was 68.9 mol %, which is consistent with the members of the genus *Lysobacter* known to have high G+C content range 61.7–70.7 mol% (Lee et al. 2006; Wei et al. 2012). The DNA–DNA relatedness values between strain THG-SKA3^T and the reference species of the genus *Lysobacter* were in the range of 20–25 % (*L. niastensis* KACC 11588^T, 24.2 ± 0.9 %; *L. panacisoli* KACC 17502^T, 21.9 ± 0.5 %). These very low DNA relatedness values suggest that THG-SKA3^T represents a novel species of the genus *Lysobacter* (Wayne et al. 1987).

Phenotypic analyses showed that strain THG-SKA3^T cells are Gram-negative, aerobic, motile by gliding, and rod shaped with size range approximately 0.2–0.5 μm in width and 1.5–2.5 μm in length (Supplementary Fig. S2). Colonies grown on R2A agar for 2 days were white, circular, smooth and have a diameter of 2–3 mm. Strain THG-SKA3^T grows on R2A, NA, TSA and LB but not on MA and MacConkey agar. Growth occurred at 18–37 °C and at pH 6.0–7.5. Optimum growth occurs at 28 °C, at pH 7.0 and in absence of NaCl. The novel isolate was able to hydrolyze L-tyrosine, starch, casein, esculin, and CMC but unable to hydrolyze DNA, Tween 80, Tween 20, urea, gelatin and chitin. Positive for nitrate reduction, oxidase and catalase tests but negative for indole production and flexirubin-pigments test. The results of biochemical and physiological tests of strain THG-SKA3^T are given in species description and in Table 1.

Both strain THG-SKA3^T and *L. niastensis* KACC 11588^T were shown to have ubiquinone 8 (O-8) as the major predominant isoprenoid quinone. This quinone system is the characteristic feature of a genus *Lysobacter*. As shown in Supplementary Fig. S3, the major polar lipids were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). These were similar to the polar lipids of *L. niastensis* KACC 11588^T that has two additional unidentified aminophospholipid (APL1-2) and an unidentified polar lipid (PL). The major fatty acids of strain THG-SKA3^T are branched chain iso-$C_{15:0}$ (19.6 %), iso-$C_{16:0}$ (22.4 %) and unsaturated iso-$C_{17:1}\omega 9c$ (17.9 %) as also has been seen in *L. niastensis* KACC 11588^T, *L. panacisoli* KACC 17502^T, *L. enzymogenes* LMG 8762^T and *L. oryzae* KCTC 22249^T. Our results are similar to the previously described cellular fatty acid profiles of other members of *Lysobacter* genus which are known to contain iso-branched chain fatty acids as major fatty acids. The cellular fatty acid profiles of strain THG-SKA3^T and the nearest reference strains are shown in Table 2.

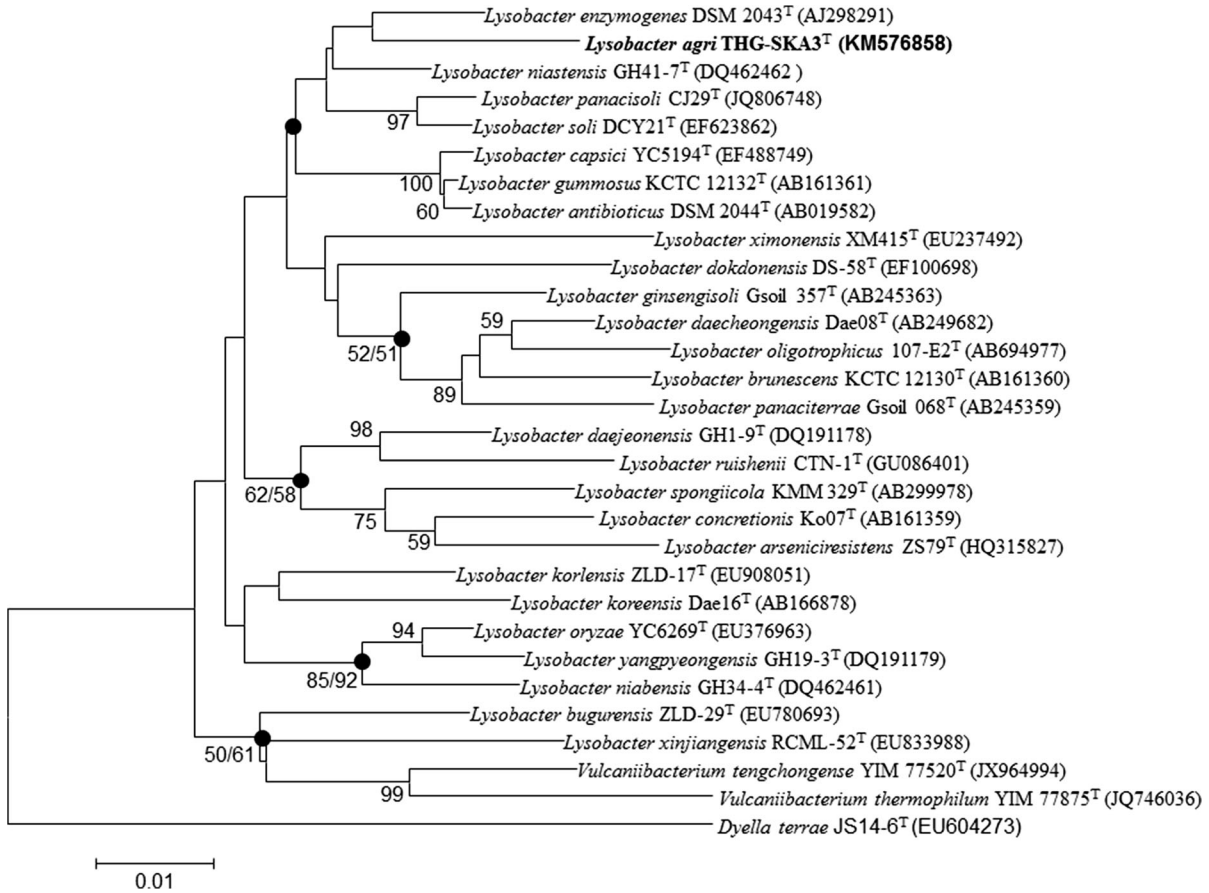

Fig. 1 The neighbor-joining tree based on 16S rRNA gene sequence analysis showing the relationships between strain THG-SKA3^T and members of the genus *Lysobacter*. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Numbers at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Bootstrap values less than 50 % were not indicated. *Dyella terrae* JS14-6^T was used as an out group. Scale bar, 0.01 substitutions per nucleotide position

On the basis part of the phylogenetic, phenotypic and chemotaxonomic data, strain THG-SKA3^T (=KACC 18283^T = CCTCC AB 2015126^T) is considered to represent a novel species of the genus *Lysobacter*, for which name *Lysobacter agri* sp. nov. is proposed.

Description of *Lysobacter agri* sp. nov

Lysobacter agri (L. gen. n. agri, of a field)

Cells are Gram-negative, aerobic, gliding and rod-shaped (0.2–0.5 μm × 1.5–2.5 μm). Colonies on R2A agar are white, circular, smooth and have a diameter of 2–3 mm. Cells grow at 18–37 °C (optimum 28 °C), at pH 6.0–7.5 (optimum pH 7.0) and in the absence of NaCl. Grow on R2A, NA, TSA and LB but not on MA and MacConkey agar. Able to hydrolyze L-tyrosine, starch, casein, esculin, and CMC but unable to hydrolyze DNA, Tween 80, Tween 20, urea, gelatin and chitin. Tests for nitrate reduction, oxidase and catalase are positive while indole production and flexirubin-pigments are negative. According to API 20 NE, positive results are obtained for the following: D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, gluconate and negative results are obtained for following: caprate, adipate, malate, citrate, phenylacetate, arginine hydrolysis and glucose acidification. Based on API ZYM test strip results, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase,

Table 2 Cellular fatty acid profiles of strain THG-SKA3^T and related species of the genus *Lysobacter*

Fatty acid	1	2	3	4	5
Straight-chain					
$C_{14:0}$	2.8	3.7	5.2	3.1	2.8
$C_{16:0}$	4.2	8.2	6.7	10.2	5.1
$C_{18:00}$	1.8	Tr	1.7	Tr	2.0
Branched-chain					
iso-$C_{11:0}$	6.1	4.5	4.2	3.9	5.2
iso-$C_{14:0}$	2.7	4.5	3.4	5.7	4.4
iso-$C_{15:0}$	19.6	18.0	17.1	15.2	14.6
iso-$C_{16:0}$	22.4	19.5	20.1	18.3	17.3
iso-$C_{17:0}$	5.3	4.8	4.0	2.2	6.1
anteiso-$C_{15:0}$	1.9	4.1	3.9	3.5	2.9
Hydroxyl					
$C_{11:0}$3OH	3.9	5.9	6.4	4.9	6.6
Unsaturated					
iso-$C_{17:1}\omega 9c$	17.9	14.3	15.6	18.8	16.4
Summed feature 3*	8.1	9.7	5.1	9.2	6.8

All data were obtained from this study. Fatty acids amounting to less than 1.0 % in all strains were not listed

Summed feature 3* consisted of $C_{16:1}\omega 6c$ and/or $C_{16:1}\omega 7c$

Strains: *1* THG-SKA3^T; *2 L. niastensis* KACC 11588^T; *3 L. panacisoli* KACC 17502^T; *3 L. enzymogenes* LMG 8762^T; *4 L. oryzae* KCTC 22249^T

Tr trace amount (<1.0 %)

cystine arylamidase, acid phosphatase, Naphthol-*AS-BI*-phosphohydrolase, α-galactosidase, *β*-galactosidase, α-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase activities are positive but trypsin, α-chymotrypsin, *β*-glucuronidase and α-fucosidase activities are negative. The predominant cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:1}\omega 9c$. The major respiratory isoprenoid quinone is ubiquinone Q-8. The predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The DNA G+C content of the type strain is 68.9 mol%.

The type strain, THG-SKA3^T (=KACC 18283^T = CCTCC AB 2015126^T), was isolated from field soil collected from Kyung Hee University, South Korea.

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