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Lysobacter humi sp. nov., a bacterium isolated from rice field

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The NCBI GenBank accession number for the 16S rRNA gene sequence of strains THG-PC4^T is KM576857.

The supplementary figures are available with the online Supplementery Material.

Abstract

A novel bacterial strain THG-PC4^T was isolated from soil sample of rice field and was characterized by using a polyphasic approach. Cells were Gram-negative, bright yellow colored, non-motile and rod-shaped. The strain was aerobic and catalase positive, optimum growth temperature and pH were 28 ℃ and 7, respectively. On the basis of 16S rRNA gene sequence analysis, strain THG-PC4^T belongs to the genus *Lysobacter* and is most closely related to *Lysobacter fragariae* KCTC 42236^T, followed by *Lysobacter oryzae* KCTC 22249^T, *Lysobacter tyrosinelyticus* KCTC 42235^T, *Lysobacter terrae* KACC 17646^T, *Lysobacter yangpyeongensis* KACC 11407^T, *Lysobacter rhizosphaerae* KCTC 42237^T and *Lysobacter niabensis* KACC 11587^T. In DNA-DNA hybridization tests, the DNA relatedness between strain THG-PC4^T and its closest phylogenetic neighbors were below 45 %. The DNA G + C content was 66.6 mol% and the predominant respiratory quinone was ubiquinone-8. Flexirubin-type pigments were found to be present. The major cellular fatty acids were iso-$C_{16:0,}$ iso-$C_{17:1}\omega 9c$, iso-$C_{17:0}$ and iso-$C_{11:0}$ 3OH. The predominant polar lipids were phosphatidylethanolamine and phosphatidylglycerol. The DNA-DNA hybridization, genotypic, chemotaxonomic and physiological data demonstrated that strain THG-PC4^T represented a novel species within the genus *Lysobacter*, for which the name *Lysobcater humi*, is proposed. The type strain is THG-PC4^T (=KACC 18284^T =CCTCC AB 2015292^T). The NCBI GenBank accession number for the 16S rRNA gene sequence of strain THG-PC4^T is KM576857.

Keywords *Lysobacter humi*, Gram-negative, 16S rRNA, Ubiquonone- 8.

Introduction

The genus *Lysobacter* was first proposed by Christensen & Cook (1978) and classified within the family *Xanthomonadaceae*. The species of the genus *Lysobacter* were isolated from various environmental sources including soil (Luo et al. 2012, Zhang et al. 2011, Jung et al. 2008, Liu et al. 2011), freshwater (Fukuda et al. 2013), rhizosphere (Aslam et al. 2009, Park et al. 2008), sludge (Bae et al. 2005, Ye et al. 2015), solid waste (Yassin et al. 2007) and deep-sea sponge (Romanenko et al. 2008). Members of the genus are Gram-staining negative, rod-shaped, have high G + C content (61.7-70.7 mol%) and contains ubiquinone 8 (Q-8) as the major respiratory quinone (Christensen & Cook 1978, Wei et al. 2012, Weon et al. 2006). Most of the members of the genus are reported to lack flagella (Lee et al. 2006, Wei et al. 2012) except *Lysobacter spongiicola* (Romanenko et al. 2008), *Lysobacter arseniciresistens* (Luo et al. 2012) and *Lysobacter mobilis* (Yang et al. 2015). Typically *Lysobacter* species shows predominance of iso-branched fatty acids and diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol as the major polar lipids (Park et al. 2008, Romanenko et al. 2008, Zhang et al. 2011, Wang et al. 2011). In this study, we report on the characterization of a novel species, *Lysobacter humi* sp. nov., designated as THG-PC4^{T}, isolated from rice field soil, by using polyphasic approach. On the basis of result obtained in this study, we propose that it should be placed in the genus *Lysobacter* as the type strain of novel species.

Methods and Materials

Bacterial isolation

Strain THG-PC4^{T} was isolated from rice field soil by plating of serial dilution method on Reasoner´s 2A agar (R2A, Difco, France). One gram of soil was suspended in 10 ml of sterile

0.85 % NaCl (w/v). Serial dilution prepared up to 10^{-5} using NaCl solution. Subsequently, 100 µl of each diluted sample was spread onto the R2A agar five times. The plates were incubated at 28 ℃ for a week. Single colonies were purified by transfer to new R2A agar plates. One isolate, THG-PC4^{T}, was selected for further study. The isolate was routinely cultured on R2A agar at 28 ℃ and preserved as a suspension in R2A broth (R2B; Difco) with glycerol (25 %, w/v) at -80 ℃. Strain THG-PC4^{T} has been deposited to the Korean Agriculture Culture Collection and China Centre for Type Culture Collection. For the comparative study, the reference strains: *L. fragariae* KCTC 42236^{T}, *L. oryzae* KCTC 22249^{T}, *L. tyrosinelyticus* KCTC 42235^{T}, *L. terrae* KACC 17646^{T}, *L. yangpyeongensis* KACC 11407^{T}, *L. rhizosphaerae* KCTC 42237^{T} and *L. niabensis* KACC 11587^{T} were obtained from the Korean Collection for Type Cultures and Korean Agricultural Culture Collection, respectively and tested using the same laboratory conditions as strain THG-PC4^{T}.

Cell growth, physiology, morphology, biochemical characteristics

Colonies of strain THG-PC4^{T} were observed after 48 h of incubation on R2A agar at 28 ℃. Suspended cells were placed on carbon- and formvar- coated nickel grids for 30 s and grids were floated on one drop of 0.1 % (w/v) aqueous uranyl acetate, blotted dry and then viewed with a transmission electron microscope (Model JEM1010; JEOL) at 11,000x magnification under standard operating conditions (Supplementary Fig. S2). Gram-staining was determined using a Gram stain Kit (bioMérieux, France) according to the manufacturer's instructions. Cells were grown in R2A broth for 24 h at 28 ℃ and then tested for gliding motility by the hanging-drop technique (Skerman 1967). Anaerobic growth was tested in serum bottles containing R2A broth supplemented with thioglycolate (0.1 %) in which the air was substituted with nitrogen gas. Production of flexirubin-type pigments was determined by the reversible color shift to red,

purple or brown when yellow or orange colonies are covered with aqueous 20 % KOH solution (Fautz & Reichenbach 1980). Catalase activity was determined by the production of bubbles from 3 % (v/v) H_2O_2 solution mixed with freshly grown cells. Oxidase activity was checked by using of 1 % (w/v) *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine reagent (Sigma, USA) according to the manufacturer's instructions. Nitrate reduction was tested in nitrate broth containing 0.2 % KNO_3 (Skerman 1967). Indole production was analyzed using Kovacs's reagent in 1 % tryptone broth (Skerman 1967). Urease activity was evaluated in Christensen's medium (Christensen 1946). Growth at different temperatures (4, 10, 15, 18, 25, 28, 30, 35, 37, 40 and 42 ℃) was accessed after 7 days of incubation on R2A agar. Different media were tested for growth such as nutrient agar (NA; Difco), tryptone soya agar (TSA, Oxoid, England), Luria Bertani agar (LA; Oxoid), R2A agar, marine agar (MA; Difco) and MacConkey Agar (Oxoid) at 28 ℃ for a week. Growth at different pH conditions (pH 4-10, at intervals of 0.5 pH units) was determined after 5 days of incubation at 28 ℃ in R2A broth. The following buffers were used to adjust pH values: citric acid/sodium citrate (pH 4-6), Na_2HPO_4/NaH_2PO_4 (pH 6-8), $Na_2CO_3/NaHCO_3$ (pH 8-10) and Na_2HPO_4/NaOH (pH 10) (Gomori 1955). The pH of the medium was confirmed after autoclaving. Tolerance for salinity was evaluated in R2A broth supplemented with [0-5 % (w/v) NaCl, at 0.5 % intervals] after 5 days of incubation at 28 ℃. Growth condition such as pH and salinity was estimated by monitoring the optical density at 600 nm. Hydrolysis of following substrate was tested using R2A agar as the basal medium: casein (2 % skim milk, Oxoid), 1 % starch (Difco), 0.1 % esculin (0.02 % ferric citrate, Difco), 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), 12 % gelatin (Sigma), 0.1 % carboxymethyl-cellulose (CMC, Sigma) and DNA [DNase agar, Scharlau (Spain), DNase activity revealed by flooding the plates

with 1N HCl] were evaluated after 5 days of incubation at 28 ℃. Enzyme activities and utilization of carbon sources were further determined by using the commercial kits API ZYM and API 20 NE, according to the manufacturer's instructions.

16S rRNA gene sequence and phylogenetic analysis

The genomic DNA of strain THG-PC4^T was extracted and purified using a commercial Genomic DNA extraction kit (Solgent, Korea). The 16S rRNA gene was amplified with the universal bacterial primer pair 27F and 1492R (Lane 1991) and the purified PCR products were sequenced by Solgent Co. Ltd (Daejeon, Korea). The identification of phylogenetic neighbors was performed using the EzTaxon-e server (Kim et al. 2012). Seq-Man software version 4.1 (DNASTAR, Inc.) was used to compile the nearly complete 16S rRNA gene sequence of strain THG-PC4^T. The multiple alignments were performed by 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). Phylogenetic trees (Fig. 1 and Supplementary Fig. S1) were constructed according to neighbor-joining (Saitou & Nei 1987) and maximum-likelihood (Felsenstein 1981) methods using the Molecular Evolutionary Genetics Analysis (MEGA 5) (Tamura et al. 2011). To evaluate the phylogenetic trees, bootstrap analysis with 1,000 sample replications were performed.

G + C content and DNA-DNA hybridization

For determination of the DNA G + C content, genomic DNA was extracted, purified by the method of Moore & Dowhan (1995) and degraded enzymatically into nucleosides (nuclease P1 and alkaline phosphatase; Sigma). The obtained nucleosides mixture was separated using a reverse-phase HPLC system (Alliance 2690 system, Waters) as described previously (Mesbah et al. 1989) with reversed-phase column SunFireTM C18 (4.6 × 250 mm × 5 μm), flow rate of 1

ml/min, solvent mixture of 200 mM $(NH_4)H_2PO_4$/acetonitrile (97 : 3, v/v) as mobile phase, and wavelength at 270 nm. The genomic DNA of *Escherichia coli* strain B (Sigma-Aldrich D4889) was used as a standard. 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 experiments were performed for strain THG-PC4^T and its closely related reference strains *L. fragariae* KCTC 42236^T, *L. oryzae* KCTC 22249^T, *L. tyrosinelyticus* KCTC 42235^T, *L. terrae* KACC 17646^T and *L. yangpyeongensis* KACC 11407^T. The optimum renaturation temperature (45 ℃) was calculated as [(0.51x G + C content) + 47] - 36 (Gillis et al. 1970), where 36 ℃ 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.

Chemotaxonomic characteristics

Polar lipids, respiratory quinone and cellular fatty acid analysis

For polar lipids and quinone analysis, freeze dried cells of strain THG-PC4^T and *L. fragariae* KCTC 42236^T were used. The polar lipids of strain THG-PC4^T and the reference strain *L. fragariae* KCTC 42236^T were extracted and analyzed. Each samples was spotted on the corner of a two-dimensional thin layer chromatography (2D-TLC) using TLC Kiesel gel 60 F_{254} plates (10 × 10 cm, Merck, USA), and developed in the first direction by using of chloroform: methanol: water (65 : 25 : 4, by vol) while in the second direction developed by chloroform: acetic acid: methanol: water (80 : 15 : 12 : 4, by vol). TLC plates were sprayed with following reagents: 5 %

molybdatophosphoric acid (total lipids, Sigma), 0.2 % ninhydrin (aminolipids, Sigma) and 2.5 % α-naphthol-sulphuric acid (glycolipids, Sigma). Then heated at 120 ℃ for 10 min. They were sprayed with molybdenum blue reagent (Sigma) for detecting phospholipids. No heating step needed for this reagent (Minnikin et al. 1984). Respiratory quinone were extracted from 200 mg freeze-dried cells with chloroform: methanol (2 : 1, v/v), separated by using hexane and eluted with hexane: diethyl ether (90 : 10, v/v), then eluent was evaporated by rotatory evaporator and dissolved in acetone, according to the method of Collins (1985). Ubiquinone purification was determined by using a reverse-phase HPLC system (Alliance 2690 system, Waters) [wavelength 270 nm, solvent MeOH: Isopropanol (7 : 5, v/v), flow rate; 1 ml/min]. For fatty acid analysis, strains were cultured on R2A agar at 28 ℃ for 2 days. Fatty acids were extracted, methylated and saponified as described by Sherlock Microbial Identification system (MIDI) and were analyzed by capillary GC (Hewlet Packard 6890) using the Microbial Identification software package with the Sherlock system MIDI 6.1 and the Sherlock Aerobic Bacterial Database (TSBA 6.1, Sasser 1990).

Results and discussion

Cell growth, morphology, physiology and biochemical tests

For strain THG-PC4^T morphology, cell growth and physiology were tested after incubation at 28 ℃ for two days, results are exposed in the description of the species and Table 1.

16S rRNA gene sequence and phylogenetic analysis

The nearly complete sequence (1,454 bp) of the 16S rRNA gene was obtained. According to the EzTaxon-e server, strain THG-PC4^T shared highest sequence similarity with *L. fragariae* KCTC 42236^T (98.1 %), followed by *L. oryzae* KCTC 22249^T (98 %), *L. tyrosinelyticus* KCTC 42235^T

(97.6 %), *L. terrae* KACC 17646^T (97.6 %), *L. yangpyeongensis* KACC 11407^T (97.2 %), *L. rhizosphaerae* KCTC 42237^T (96.6 %) and *L. niabensis* KACC 11587^T (96.5 %). In addition, they also showed low sequence similarities (< 96 %) with other species of the family *Xanthomonadaceae.* The neighbor-joining tree showed that the strain THG-PC4^T clustered within the genus *Lysobacter*. This tree also supported the tree generated by maximum-likelihood algorithms with high bootstrap values. These results indicated that strain THG-PC4^T is clearly grouped within the genus *Lysobacter.*

DNA G + C content mol % analysis and DNA-DNA relatedness

The DNA G + C content of strain THG-PC4^T was 66.6 mol%. Levels of DNA-DNA relatedness between strain THG-PC4^T and *L. fragariae* KCTC 42236^T, *L. oryzae* KCTC 22249^T, *L. tyrosinelyticus* KCTC 42235^T, *L. terrae* KACC 17646^T and *L. yangpyeongensis* KACC 11407^T were 41±0.5 %, 35±1 %, 31.5±0.5 %, 24±1.5 %, 20.5±0.5 %, respectively. The DNA-DNA relatedness values were significantly lower than the threshold value of 70 % recommended for recognition of separate species (Wayne et al. 1987). As low sequence similarities (< 97 %) were found between strain THG-PC4^T and all other species (except *L. fragariae* KCTC 42236^T, *L. oryzae* KCTC 22249^T, *L. tyrosinelyticus* KCTC 42235^T, *L. terrae* KACC 17646^T and *L. yangpyeongensis* KACC 11407^T) of the genus *Lysobacter* with validly published names (Stackebrandt & Goebel 1994), DNA-DNA hybridization studies of strain THG-PC4^T with other relatives were not performed.

Polar lipid, quinone and fatty acid analysis

The major polar lipids of strain THG-PC4^T were phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The presence of two unidentified lipids (L1, L3) and absence of diphosphatidylglycerol (DPG), an unidentified lipid (L5) distinguished the polar lipid profile of

strain THG-PC4^T from its closest phylogenetic relative *L. fragariae* KCTC 42236^T (Fig. S3). The major isoprenoid quinone of strain THG-PC4^T was Q-8, which is consistent with genus *Lysobacter*. The major cellular fatty acids (> 10 %) were iso-$C_{16:0}$ (23.3 %), iso-$C_{17:1}\omega 9c$ (17.1 %), iso-$C_{17:0}$ (14.3 %) and iso-$C_{11:0}$ 3 OH (10.2 %) (Table 2).

The physiological, biochemical and morphological characteristics of strain THG-PC4^T are given in the species description and Table 1. The phylogenetic inference is supported by the unique combination of chemotaxonomic and biochemical characteristics of the novel strain. Hence, it can be concluded that the strain THG-PC4^T constitutes a novel member of the genus *Lysobacter* Therefore, the name of the strain HG-PC4^T (=KACC 18284^T =CCTCC AB 2015292^T) *Lysobcater humi* sp. nov., is proposed.

Description of *Lysobacter humi* sp. nov

Lysobacter humi (huˈmi. L. gen. n. *humi* of/from soil).

Cells are Gram-negative, aerobic, non-motile and rod-shaped. Colonies on R2A agar are bright yellow colored and circular. Cells grow at 18-37 ℃ (optimum 28 ℃); at pH 6.5-7.5 (optimum pH 7) and at 0-1 % NaCl (optimum 0 % NaCl). Catalase-positive and oxidase-negative. Flexirubin-type pigments are present. Growth occurs on R2A agar, NA and MacConkey agar, but not on LA, MA and TSA. Positive for hydrolysis of casein, L-tyrosine, Tween 80, gelatin and esculin but negative for hydrolysis of starch, CMC, chitin and DNA. Tests for nitrate reduction and indole production are negative. Negative results obtained for both glucose fermentation and acidification. Positive for following enzyme activities: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-

chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *β*-galactosidase, *α*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, arginine dihydrolase and urease; negative for following: lipase, *α*-galactosidase, *β*-glucuronidase, *α*-mannosidase and *α*-fucosidase. The adipic acid is utilized; D-mannose and gluconate were weakly utilized. The following compounds are not utilized as sole carbon source: L-arabinose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, capric acid, malic acid, triosodium citrate and phenylacetic acid. The major isoprenoid quinone is ubiquinone-8. The major polar lipids are phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The major cellular fatty acids are iso-$C_{16:0,}$ iso-$C_{17:1}\omega 9c$, iso-$C_{17:0}$ and iso-$C_{11:0}$ 3OH. The DNA G + C content of the type strain is 66.6 mol%.

The type strain, THG-PC4^T (=KACC 18284^T =CCTCC AB 2015292^T), was isolated from soil of rice field, Republic of Korea.

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Table 1. The biochemical and physiological characteristics of strain THG-PC4^T and the reference strains of genus *Lysobacter*.

Strains: 1, THG-PC4^T; 2, *L. fragariae* KCTC 42236^T; 3, *L. oryzae* KCTC 22249^T; 4, *L. tyrosinelyticus* KCTC 42235^T; 5, *L. terrae* KACC 17646^T; 6, *L. yangpyeongensis* KACC 11407^T; 7, *L. rhizosphaerae* KCTC 42237^T; 8, *L. niabensis* KACC 11587^T.

All data were obtained from this work. All strains are observed to grow on R2A agar plates. All strains are positive for: hydrolysis of casein and gelatin; enzyme activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase. All strains are negative for: Gram-reaction; hydrolysis of chitin; indole production; both glucose fermentation and acidification; enzyme activity of α-fucosidase; utilization of L-arabinose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, capric acid, malic acid, triosodium citrate and phenylacetic acid.

Table 2. Fatty acid profiles of strain THG-PC4^T and related species of the genus *Lysobacter*.

Strains: 1, THG-PC4^T; 2, *L. fragariae* KCTC 42236^T; 3, *L. oryzae* KCTC 22249^T; 4, *L. tyrosinelyticus* KCTC 42235^T; 5, *L. terrae* KACC 17646^T; 6, *L. yangpyeongensis* KACC 11407^T; 7, *L. rhizosphaerae* KCTC 42237^T; 8, *L. niabensis* KACC 11587^T.

All the data are from this study. For fatty acid analysis all strains were cultured on R2A agar at 28 ℃ for 48 h. Fatty acids of less than 0.5 % in all strains are not listed. tr: traces (< 1 %).

Figure legends

Fig. 1. The neighbor-joining (NJ) tree based on 16S rRNA gene sequence analysis showing phylogenetic relationships of strain THG-PC4^T and members of the genus *Lysobacter*. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. Bootstrap values more than 70 % based on 1,000 replications are shown at branching points. Scale bar, 0.005 substitutions per nucleotide position.

Supplementary Fig. S1. The maximum-likelihood (ML) tree based on 16S rRNA gene sequence analysis showing phylogenetic relationships of strain THG-PC4^T and members of the genus *Lysobacter*, values less than 50 % were not shown.

Supplementary Fig. S2. Transmission electron micrograph of strain THG-PC4^T after negative staining with uranyl acetate, Bar, 0.2 μm.

Supplementary Fig. S3. Two-dimensional thin-layer chromatography of the total polar lipids of THG-PC4^T (a) and *Lysobacter fragariae* KCTC 42236^T (b), stained for total polar lipids with 5 % ethanolic molybdophosphoric acid. Abbreviations: PE, phosphatidylethanolamine; PME, phosphotidylmethyletanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PL, unidentified phospholipid; L1-5, unidentified polar lipids.