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Lysobacter hankyongensis sp. nov., isolated from activated sludge and *Lysobacter sediminicola* sp. nov., isolated from freshwater sediment

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Two novel strains, designated KTCe-2^T and 7C-9^T, isolated from an activated sludge and freshwater sediment, respectively in South Korea, were characterized by a polyphasic approach to clarify their taxonomic positions. Phylogenetic analysis based on 16S rRNA gene sequences indicated that both isolates belong to the genus *Lysobacter* and are most closely related to '*Lysobacter daecheongensis*' Dae 08 (98.5 % and 97.6 % similarity for strains KTCe-2^T and 7C-9^T, respectively), *Lysobacter brunescens* KCTC 12130^T (98.4 % and 97.2 %), and *Lysobacter oligotrophicus* JCM 18257^T (97.1 % and 96.8 %). The G+C content of the genomic DNA of strains KTCe-2^T and 7C-9^T was 68.6 % and 71.5 mol%, respectively. Strains KTCe-2^T and 7C-9^T possessed ubiquinone-8 as the sole respiratory quinone, and a fatty acid profile with iso-$C_{15:0}$ and iso-$C_{16:0}$ as the major fatty acids supported the affiliation of the two strains to the genus *Lysobacter*. Moreover, the physiological and biochemical results and low DNA–DNA relatedness values allowed the phenotypic and genotypic differentiation of strains KTCe-2^T and 7C-9^T from other species of the genus *Lysobacter* with validly published names. Therefore, the two isolates represent two novel species of the genus *Lysobacter*, for which the name *Lysobacter hankyongensis* sp. nov. (type strain KTCe-2^T=JCM 18204^T=KACC 16618^T) and *Lysobacter sediminicola* sp. nov. (type strain 7C-9^T=JCM 18205^T=KACC 16617^T) are proposed.

The genus *Lysobacter* was first described by Christensen & Cook (1978) and the description was emended by Park *et al.* (2008). Members of the genus *Lysobacter*, in the family *Xanthomonadaceae*, contain ubiquinone Q-8 as the major respiratory quinone and have a high DNA G+C content (Park *et al.*, 2008; Wang *et al.*, 2009). Species of the genus *Lysobacter* are Gram-negative, aerobic, non-fruiting, gliding organisms and with colonies that are very mucoid and cream, pink or yellow–brownish in colour. Members of the genus *Lysobacter* can also be separated from other related microbes due to high genomic G+C content (usually ranging between 65–72 mol%), lytic activity against other micro-organisms, and the lack of flagella (Sullivan *et al.*, 2003). Members of the genus were found to have great potential antibiotic compounds against human pathogens (Ahmed *et al.*, 2003; Hashizume *et al.*, 2004) and biocontrol agents for plant fungal pathogens (Islam *et al.*, 2005; Park *et al.*, 2008). At the time of writing, there are 33 recognized species of the genus *Lysobacter* (http://www.bacterio.net) including the recently described *Lysobacter panacisoli* (Choi *et al.*, 2014), *Lysobacter terrae* (Ngo *et al.*, 2015), *Lysobacter caeni* (Ye *et al.*, 2015), *Lysobacter mobilis* (Yang *et al.*, 2015) and *Lysobacter novalis* (Singh *et al.*, 2015a). In addition, another recently described species that has not yet been validly published is '*Lysobacter agri*' (Singh *et al.*, 2015b).

In this study, we report on the taxonomic characterization of two strains, designated KTCe-2^T, and 7C-9^T, which appeared to be members of the genus *Lysobacter*. Strains KTCe-2^T and 7C-9^T were routinely cultured on R2A agar plates at 30 °C and preserved at −70 °C as a suspension in R2A broth containing 20 % (w/v) glycerol.

Extraction of genomic DNA was performed with a commercial genomic DNA extraction kit (Solgent), and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KTCe-2^T and 7C-9^T are JQ349049 and JQ349048, respectively.

Three supplementary figures and a supplementary table are available with the online Supplementary Material.

according to Im *et al.* (2010). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database or the EzTaxon server (http://www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012). Multiple alignments were performed using the CLUSTAL X program (Thompson *et al.*, 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using Kimura's two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed by using neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971), and maximum-likelihood methods in the MEGA5 program (Tamura *et al.*, 2011) with bootstrap values based on 1000 replications (Felsenstein, 1985).

Nearly complete 16S rRNA gene sequences of strains KTCe-2^T (1472 nt) and 7C-9^T (1469 nt) were determined and subjected to comparative analysis. Phylogenetic analysis using the neighbour-joining method based on 16S rRNA gene sequences indicated that strains KTCe-2^T and 7C-9^T are clustered within the genus *Lysobacter* (Fig. 1). Moreover, this relationship was also evident in the phylogenetic tree based on the maximum-likelihood method (Fig. S1, available in the online Supplementary Material). The highest degrees of 16S rRNA gene sequence similarity determined were to '*Lysobacter daecheongensis*' Dae08 (98.5 % and 97.6 % for strains KTCe-2^T and 7C-9^T, respectively), *Lysobacter brunescens* KCTC 12130^T (98.4 % and 97.2 %), *Lysobacter oligotrophicus* 107-E2^T (97.1 %

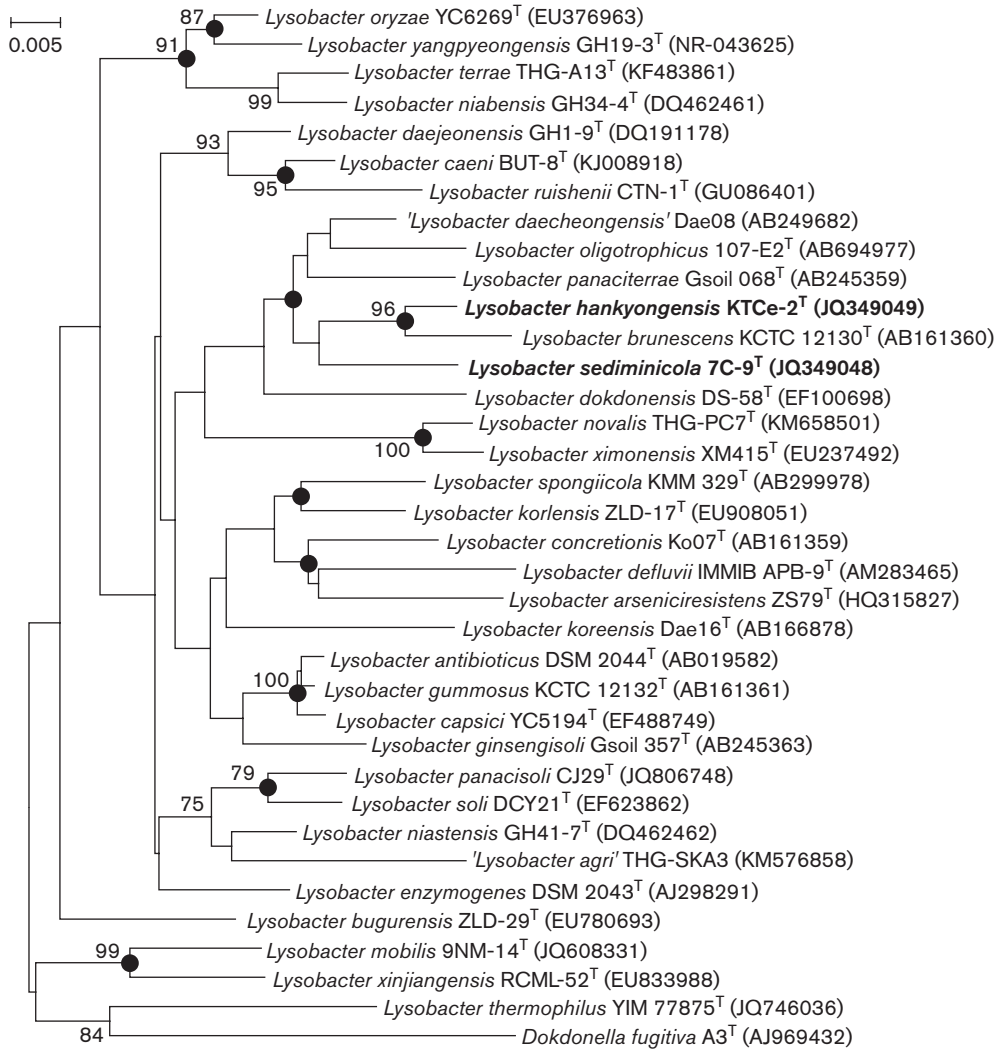

Fig. 1. Phylogenetic relationships of strains KTCe-2^T and 7C-9^T with species of the genus *Lysobacter* and other related species. The tree was reconstructed by using the neighbour-joining method based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) >70 % are shown at branch points. *Dokdonella fugitiva* A3^T (GenBank accession no. AJ969432) was used as an outgroup. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with maximum-parsimony and maximum-likelihood algorithms. Bar, 0.005 substitutions per nucleotide position.

and 96.8 %) and *Lysobacter panaciterrae* Gsoil 068^T (97.0 % and 96.4 %). Strains KTCe-2^T and 7C-9^T showed 97.0 % 16S rRNA gene sequence similarity between each other, suggesting that they are differentiated at the species level (Stackebrandt & Goebel, 1994).

On the basis of these phylogenetic results, '*L. daecheongensis*' KCTC 12600, *L. brunescens* KCTC 12130^T, *L. oligotrophicus* JCM 18257^T and *L. panaciterrae* KCTC 12601^T were selected as the closest recognized neighbours of strains KTCe-2^T and 7C-9^T and were obtained from the respective culture collections, grown under the same conditions and used as reference strains in most of the subsequent phenotypic tests.

The Gram reaction was determined using the non-staining method, as described by Buck (1982). Cell morphology was examined with a scanning electron microscope (SU-3500; Hitachi), using cells grown for 2 days at 30 °C on R2A agar. Cell motility was determined using the hanging-drop method. Catalase and oxidase tests were performed as outlined by Cappuccino & Sherman (2002). Biochemical tests were carried out by using API 20NE, API ID 32GN and API ZYM kits according to the instructions of the manufacturer (bioMérieux). Tests for degradation of DNA (using DNase agar from Scharlau, with DNase activity by flooding plates with 1 M HCl), casein, starch (Atlas, 1993), olive oil (Kouker & Jaeger, 1987), xylan and carboxylmethyl-cellulose (Ten *et al.*, 2004) were performed and evaluated after 7 days. Growth at different temperatures (4, 10, 15, 25, 30, 37 and 42 °C) and pH (pH 4–10.0 at intervals of 0.5 pH units) was assessed after incubation for 5 days at 30 °C. Three different buffers (final concentration, 50 mM) were used to adjust the pH of R2A broth: acetate buffer was used for pH 4.0–5.5, phosphate buffer was used for pH 6.0–8.0 and Tris buffer was used for pH 8.5–10.0. Salt tolerance was tested on R2A medium supplemented with 1–10 % NaCl (w/v, at intervals of 1 %) and growth was assessed after incubation for 7 days. Growth on nutrient agar (NA; BD), trypticase soy agar (TSA; BD), LB agar (BD) and MacConkey agar (BD) was also evaluated at 30 °C.

Cells of both strain KTCe-2^T and strain 7C-9^T were Gram-reaction-negative, aerobic, non-motile, non-spore-forming, and rod-shaped (Fig. S2). Colonies of strain KTCe-2^T grown on R2A agar were circular, convex, translucent, and yellow–orange turning to dark orange after incubation for 48 h at 30 °C. Colonies of strain 7C-9^T were slightly different, in being yellowish and not becoming dark orange after a long incubation time. Neither strain grew on TSA, LB, DNase or MacConkey agar, whereas both strains grew weakly on NA at 30 °C. Physiological characteristics of strains KTCe-2^T and 7C-9^T are summarized in the species descriptions and a comparison of selective characteristics of strains KTCe-2^T and 7C-9^T and the reference strains are given in Table 1.

For the measurement of the DNA G+C content, genomic DNA of the novel strains were extracted and purified as described by Moore & Dowhan (1995), then enzymically degraded into nucleosides, and the G+C content was determined as described by Mesbah *et al.* (1989) using a reverse-phase HPLC. Strains KTCe-2^T and 7C-9^T were investigated for their polar lipid contents; the polar lipids were extracted from 50 mg freeze-dried cells, examined by two-dimensional TLC and identified as described by Minnikin *et al.* (1984). Isoprenoid quinones were extracted with chloroform/methanol (2 : 1, v/v), evaporated under vacuum conditions, and re-extracted in n-hexane/water (1 : 1, v/v). The crude n-hexane/quinone solution was purified using Sep-Pak silica Vac cartridges (Waters) and subsequently analysed by HPLC as previously described (Hiraishi *et al.*, 1996). Cellular fatty acid profiles were determined for strains grown on R2A agar for 2 days at 30 °C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids, analysed with a gas chromatograph (6890; Hewlett Packard), were identified by the Microbial Identification software package based on Sherlock Aerobic Bacterial Database (TSBA60) (Sasser, 1990). DNA–DNA hybridization experiments were performed between strains KTCe-2^T and 7C-9^T and the two reference strains *L. brunescens* KCTC 12130^T and '*L. daecheongensis*' Dae08 according to the method described by Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and micro-dilution wells.

The DNA G+C contents of the strains KTCe-2^T and 7C-9^T were 68.6 and 71.5 mol%, respectively which were similar to those of '*L. daecheongensis*' KCTC 12600, *L. brunescens* DSM 6979^T, *L. oligotrophicus* JCM 18257^T and *L. panaciterrae* DSM 17927^T (Table 1). The major polar lipids detected in strain KTCe-2^T were phosphatidylglycerol and phosphatidylethanolamine, while in strain 7C-9^T contained diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine (Fig. S3). The sole respiratory quinone for both strains KTCe-2^T and 7C-9^T was ubiquinone-8 (Q-8). The fatty acid profiles of strains KTCe-2^T and 7C-9^T were compared with those of the reference strains. The major fatty acids of strain KTCe-2^T were iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{14:0}$ and summed feature 3 ($C_{16:1}\omega6c$/$C_{16:1}\omega7c$), while strain 7C-9^T had the major fatty acids iso-$C_{15:0}$, iso-$C_{16:0}$ and summed feature 9 (iso-$C_{17:1}\omega9c$/$C_{16:0}$ 10-methyl), a typical profile of members of the genus *Lysobacter* (Bae *et al.*, 2005; Weon *et al.*, 2006, 2007; Romanenko *et al.*, 2008). However, some qualitative and quantitative differences in the fatty acid profiles distinguished strains KTCe-2^T and 7C-9^T from each other and from other species of the genus *Lysobacter* (Table S1). DNA–DNA relatedness values between strain KTCe-2^T and *L. brunescens* KCTC 12130^T and '*L. daecheongensis*' KCTC 12600 were 31.4±1.5 % and 23.6±2.4 %, respectively. Similarly, DNA–DNA relatedness values between strain 7C-9^T and *L. brunescens* KCTC 12130^T and '*L. daecheongensis*' KCTC 12600 were 8.4±1.9 % and 5.7±0.7 %, respectively. According to Wayne *et al.* (1987), a DNA–DNA relatedness value of

Table 1. Phenotypic characteristics of strains KTCe-2^T and 7C-9^T, and related species of the genus *Lysobacter*

Strains: 1, *L. hankyongensis* sp. nov. KTCe-2^T; 2, *L. sediminicola* sp. nov. 7C-9^T; 3, '*L. daecheongensis*' KCTC 12600; 4, *L. brunescens* KCTC 12130^T; 5, *L. oligotrophicus* JCM 18257^T; 6, *L. panaciterrae* KCTC 12601^T. All data are from this study except where otherwise indicated. All strains were positive for gelatin hydrolysis and alkaline phosphatase activity. All strains were negative for indole production, arginine dihydrolase and *N*-acetyl-D-glucosaminidase activity, and assimilation of phenylacetic acid, malic acid, L-alanine and capric acid. +, Positive; −, negative.

Characteristic	1	2	3	4	5	6
Isolation source	Sludge	Sediment of freshwater	Sediment of stream	Soil	Freshwater	Soil
Temperature range for growth (°C)	10–30	25–30	20–30*[a]	4–50[b]	5–25[c]	15–45[d]
Hydrolysis of:						
Urease	+	+	+	+	−	−
Aesculin	+	−	−	+	+	+
Enzyme activity						
Nitrate reduction	−	−	−	−	−	+
Esterase	−	+	−	+	−	+
Esterase lipase	−	+	+	−	−	+
Lipase	−	+	−	−	−	+
Leucine arylamidase	+	−	+	−	−	+
Valine arylamidase	−	−	−	−	+	+
Cystine arylamidase	+	+	−	−	−	+
Trypsin	+	−	−	+	+	+
α-Chymotrypsin	+	−	−	−	+	+
Acid phosphatase	−	−	+	+	+	+
Naphthol-AS-BI-phosphohydrolase	−	−	+	+	+	+
α-Galactosidase	−	+	−	−	−	+
β-Galactosidase	−	+	−	−	−	−
α-Glucosidase	−	+	−	−	−	−
β-Glucosidase	−	+	−	−	+	−
α-Fucosidase	−	+	−	−	−	−
Assimilation of:						
D-Glucose	+	−	−	+	−	+
D-Mannose	−	−	−	+	−	+
D-Mannitol	+	−	−	−	−	+
Maltose	+	+	−	+	−	−
N-Acetylglucosamine	+	−	−	−	−	+
Potassium gluconate	−	+	−	−	−	−
Capric acid	−	−	+	−	−	−
Adipic acid	−	+	−	−	+	−
L-Rhamnose	−	−	−	−	+	−
D-Ribose	+	−	−	−	−	+
Inositol	−	−	−	−	+	−
D-Saccharose	+	+	−	−	−	+
Itaconic acid	+	+	−	−	+	−
Suberic acid	+	+	−	−	+	−
Sodium malonate	+	−	−	−	+	−
Sodium acetate	−	+	−	−	+	+
Lactic acid	+	−	−	−	+	−
Potassium 5-gluconate	−	+	−	−	+	−
Glycogen	+	−	−	−	−	+
3-Hydroxybenzoic acid	+	−	−	−	+	−
L-Serine	+	−	−	−	+	+
Salicin	+	−	−	−	+	+
Melibiose	+	−	−	−	+	−
L-Fucose	+	−	−	−	+	+
D-Sorbitol	+	−	−	+	+	−
L-Arabinose	+	−	−	−	+	−
Propionic acid	+	−	−	−	+	+
Valeric acid	+	+	−	−	+	+

Table 1. cont.

Characteristic	1	2	3	4	5	6
Trisodium Citrate	+	−	−	−	+	−
L-Histidine	+	−	−	−	−	−
Potassium 2-gluconate	+	−	−	−	+	−
3-Hydroxybutyric acid	+	−	−	−	+	+
4-Hydroxybenzoic acid	+	+	−	−	+	−
L-Proline	+	+	−	−	+	−
DNA G+C content (mol%)	68.6	71.5	69.3[a]	67.7[b]	66.1[c]	67[d]

*Data from: *a*, Ten *et al.* (2008); *b*, Christensen & Cook (1978) for *L. brunescens* DSM 6979^T; *c*, Fukuda *et al.* (2013); *d*, Ten *et al.* (2009) for *L. panaciterrae* DSM 17927^T.

70 % is considered the threshold value for the delineation of genospecies, so the results obtained are low enough to assign strains KTCe-2^T and 7C-9^T as novel species of the genus *Lysobacter*.

On the basis of the data and observations described above, it is appropriate to conclude that strains KTCe-2^T and 7C-9^T should be assigned to the genus *Lysobacter* as the type strains of two novel species, for which the names *Lysobacter hankyongensis* sp. nov. and *Lysobacter sediminicola* sp. nov., respectively, are proposed.

Description of *Lysobacter hankyongensis* sp. nov.

Lysobacter hankyongensis (han.kyong.en′sis. N.L. masc. adj. *hankyongensis* pertaining to Hankyong National University Republic of Korea, where taxonomic studies of this taxon were performed).

Cells are Gram-reaction-negative, aerobic, non-motile, non-spore-forming and rod-shaped (0.3–0.4 μm in diameter and 1.0–1.5 μm in length). Colonies grown on R2A agar for 2 days are 0.5–2 mm in diameter, smooth, circular, convex, translucent and yellow–orange turning dark orange after incubation for 48 h at 30 °C. Catalase-positive and oxidase-negative. Growth occurs at 10–30 °C and pH 6–9, but growth is not observed below 10 °C or above 30 °C. Optimum growth occurs at 30 °C and pH 6.0–7.0 without additional NaCl supplement. Growth is inhibited in the presence of 1.0 % (w/v) NaCl. Hydrolyses gelatin and starch, but not casein, CM-cellulose, xylan, olive oil or DNA. Nitrate is not reduced to nitrite. The substrates utilized as sole carbon sources (API ID 32 GN, API 20 NE) and the enzyme activities (API ZYM) are listed in Table 1. The major polar lipids are phosphatidylglycerol and phosphatidylethanolamine, while the minor polar lipids are an unidentified phospholipid and two unidentified polar lipids (L1 and L2). Ubiquinone-8 (Q-8) is the sole respiratory quinone. The major fatty acids are summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), iso-$C_{16:0}$, iso-$C_{15:0}$ and iso-$C_{14:0}$.

The type strain, strain KTCe-2^T (=JCM 18204^T=KACC 16618^T), was isolated from activated sludge in Daejeon city, Republic of Korea. The G+C content of genomic DNA of the type strain is 68.6 mol%.

Description of *Lysobacter sediminicola* sp. nov.

Lysobacter sediminicola (se.di.mi.ni′co.la. L. n. *sedimen*, *-inis* sediment; L. suff. *-cola* inhabitant, dweller; N.L. n. *sediminicola* sediment-dweller, referring to the source of the type strain).

Cells are Gram-reaction-negative, aerobic, non-motile, non-spore-forming and rod-shaped (0.4–0.6 μm in diameter and 1.5–3.0 μm in length). Colonies grown on R2A agar for 2 days are 0.5–2 mm in diameter, smooth, circular, convex, translucent and yellowish. Catalase- and oxidase-negative. The optimum growth temperature and pH are 30 °C and pH 6.5–7.5 without additional NaCl supplement. Growth is inhibited in the presence of 0.5 % (w/v) NaCl. Hydrolyses gelatin and starch, but not aesculin, casein, CM-cellulose, xylan, olive oil or DNA. Nitrate is not reduced to nitrite. The substrates utilized as sole carbon sources (API ID 32 GN, API 20 NE) and the enzyme activities (API ZYM) are listed in Table 1. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine, and the minor polar lipids are an unidentified phospholipid, two unidentified aminolipids and an unknown polar lipid. The sole respiratory quinone is ubiquinone-8 (Q-8). The major fatty acids are iso-$C_{15:0}$, summed feature 9 (iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl), and iso-$C_{16:0}$.

The type strain, 7C-9^T (=JCM 18205^T=KACC 16617^T), was isolated from freshwater sediment flowed from Gapcheon stream, Daejeon City, South Korea. The DNA G+C content of the type strain is 71.5 mol%.

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