International Journal of Systematic and Evolutionary Microbiology (2015), 65, 845–850 DOI 10.1099/ijs.0.000024

Lysobacter caeni sp. nov., isolated from the sludge of a pesticide manufacturing factory

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Strain BUT-8^T, a Gram-stain-negative, non-motile and rod-shaped aerobic bacterium, was isolated from the activated sludge of a herbicide-manufacturing wastewater treatment facility. Comparative 16S rRNA gene sequence analysis revealed that strain BUT-8^T clustered with species of the genus *Lysobacter* and was closely related to *Lysobacter ruishenii* DSM 22393^T (98.3 %) and *Lysobacter daejeonensis* KACC 11406^T (98.7 %). The DNA G+C content of the genomic DNA was 70.6 mol%. The major respiratory quinone was ubiquinone-8, and the major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and an aminolipid. The major cellular fatty acids were iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, iso-$C_{11:0}$, iso-$C_{11:0}$ 3OH and summed feature 9 (comprising iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$10-methyl). The DNA–DNA relatedness between strain BUT-8^T and its closest phylogenetic neighbours was below 70 %. Phylogenetic, chemotaxonomic and phenotypic results clearly demonstrated that strain BUT-8^T belongs to the genus *Lysobacter* and represents a novel species for which the name *Lysobacter caeni* sp. nov. is proposed. The type strain is BUT-8^T (=CCTCC AB 2013087^T=KACC 17141^T).

The genus *Lysobacter*, which was first described by Christensen & Cook (1978), belongs to the family *Xanthomonadaceae* vwithin the class *Gammaproteobacteria*. At the time of writing, the genus *Lysobacter* consists of 27 species with validly published names (http://www.bacterio.net/lysobacter.html). Most of the species of the genus *Lysobacter* have been isolated from soils; however, some were isolated from other environmental samples, e.g. *Lysobacter concretionis* (Bae *et al.*, 2005) was isolated from anaerobic granules in an upflow anaerobic sludge blanket reactor, *Lysobacter defluvii* (Yassin *et al.*, 2007) was isolated from municipal solid waste, *Lysobacter oligotrophicus* (Fukuda *et al.*, 2013) was isolated from a freshwater lake in Antarctica, and *Lysobacter spongiicola* (Romanenko *et al.*, 2008) was isolated from a deep-sea sponge. Strain BUT-8^T was originally isolated from the activated sludge of a herbicide-manufacturing wastewater treatment facility in Kunshan city, Jiangsu Province, PR China. In the present study, the taxonomic position of strain BUT-8^T was determined.

Lysobacter daejeonensis KACC 11406^T (Weon *et al.*, 2006) and *Lysobacter ruishenii* DSM 22393^T (Wang *et al.*, 2011), which showed the highest 16s rRNA gene sequence similarities (>97 %) with BUT-8^T, were used as reference strains for phenotypic characterization. Unless indicated otherwise, the morphological, physiological and biochemical characteristics of strain BUT-8^T and the reference strains were determined using routine cultivation on Trypticase Soya Agar (TSA; Difco) or Trypticase Soya broth (TSB; Difco) at 30 °C.

Cellular morphology was observed during the exponential growth phase under phase-contrast microscopy (Nikon inverted research microscope Eclipse Ti) and transmission electron microscopy (Hitachi, H-7650). Motility was studied by the hanging-drop method (Bernardet *et al.*, 2002). Gram staining was performed according to the classical Gram procedure (Buck, 1982) and further confirmed by the conventional Gram-staining method (Smibert *et al.*, 1994). Endospore formation was detected from

Abbreviations: AL, aminolipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BUT-8^T is KJ008918.

Five supplementary figures are available with the online Supplementary Material.

malachite green staining. Growth at various temperatures (5, 10, 15, 20, 25, 28, 30, 37, 40, 42 and 45 °C), in various salt concentrations [0–7 % (w/v) NaCl (increments of 0.5 %)] and at various pH values [The pH was maintained using three different buffers (final concentration, 50 mM): sodium acetate buffer (for pH 4.0–5.5), sodium phosphate buffer (for pH 6.0–8.0) and Tris/HCl buffer (for pH 8.5–10.0)]. Oxidase and catalase activities were determined using oxidase discs and 3 % (v/v) H_2O_2, according to methods described by Smibert *et al.* (1994). API 20NE, API 32GN and API ZYM kits (bioMérieux) were used to determine biochemical properties according to the manufacturer's instructions.

Cells of strain BUT-8^T were Gram-stain-negative, non-spore-forming, non-motile, aerobic rods, 0.42–0.57 μm × 1.76–2.0 μm l (see Fig. S1, available in the online Supplementary Material). Colonies were yellow-green, convex and circular. Strain BUT-8^T grew at 15–37 °C (optimum 28–30 °C), pH 6.0–9.0 (optimum pH 7.0) and at an NaCl concentration of 0–1 % (w/v) (optimum 0.5 %). Strain BUT-8^T was positive for oxidase and catalase. The differential phenotypic and biochemical characteristics of strain BUT-8^T and the type strains of recognized species of the genus *Lysobacter* are summarized in Table 1.

Genomic DNA was extracted according to standard procedures (Sambrook & Russell, 2001). The nearly complete 16S rRNA gene sequence was obtained by PCR amplification using a set of universal primers, 5′-AGAGTTTGATCCTGGCTCAG-3′ (*Escherichia coli* bases 8–27) and 5′-TACCTTGTTACGACTT-3′ (*Escherichia coli* bases 1507–1492), and then sequenced with an automatic sequencer (Applied Biosystem, model 3730). A similarity-based search was performed using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The 16S rRNA gene sequence of strain BUT-8^T was aligned with those of the type strains of recognized species of the genus *Lysobacter* using the CLUSTAL_X program (Thompson *et al.*, 1997). Phylogenetic analysis was performed using MEGA3 (Kumar *et al.*, 2004). An evolutionary distance matrix was calculated by the Kimura two-parameter distance model (Kimura, 1980), and phylogenetic trees were reconstructed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods; the robustness of trees was examined using bootstrap analysis of 1000 replicates (Felsenstein, 1985).

An almost-complete 16S rRNA gene sequence (1506 bp) was determined for the strain. Sequence BLAST analysis revealed that strain BUT-8^T was a member of the genus *Lysobacter* and was most closely related to *L. ruishenii* DSM 22393^T (98.3 %) and *L. daejeonensis* KACC 11406^T (98.7 %). Less than 97.0 % 16S rRNA gene sequence similarity was observed with other members of the

Table 1. Characteristics that differentiate strain BUT-8^T from phylogenetically related species of the genus *Lysobacter*

Strains: 1, BUT-8^T (data from this study); 2, *L. ruishenii* DSM 22393^T (data from this study, except for the DNA G+C content which is from Wang *et al.*, 2011); 3, *L. daejeonensis* KACC 11406^T (data from this study, except for the DNA G+C content which is from Weon *et al.*, 2006); 4, *L. niastensis* DSM 18481^T (Weon *et al.*, 2007; Ten *et al.*, 2009); 5, *L. soli* DCY21^T (*Srinivasan et al.*, 2010); 6, *L. enzymogenes* DSM 2043^T (Bae *et al.*, 2005; Ten *et al.*, 2009; Wang *et al.*, 2009). +, Positive; (+), weakly positive; −, negative; NA, data not available.

Characteristic	1	2	3	4	5	6
Gliding motility	−	+	−	+	+	+
Colony colour	yellow-green	yellow	yellow	Light beige	yellow	deep yellow-cream
Growth temperature (°C)	15–37	15–37	10–37	10–40	4–42	NA
NaCl tolerance (%, w/v)	0–1	0–1	0–3	0–1	NA	0–1
pH range	6.0–9.0	6.0–9.0	6–8	4–9	5–10.5	NA
Catalase	+	+	−	+	+	+
β-Galactosidase	+	−	−	+	−	+
Arginine dihydrolase	−	+	−	−	NA	−
Urease	+	−	(+)	−	NA	−
Assimilation of						
N-Acetylglucosamine	−	+	−	+	+	+
D-Glucose	−	+	+	(+)	+	+
3-Hydroxybenzoic acid	−	+	(+)	−	+	−
4-Hydroxybenzoic acid	−	+	(+)	−	+	−
Malate	+	+	−	−	−	+
Maltose	−	−	+	+	+	+
Mannose	+	−	−	−	+	+
Potassium 5-Ketogluconate	−	+	−	−	−	−
L-Serine	−	+	−	−	−	+
Suberic acid	−	+	+	−	−	−
Valerate	−	+	+	−	−	+
DNA G+C content (mol%)	70.6	67.1	61.7	66.6	65.4	69.0

genus *Lysobacter*. In the neighbour-joining tree (Fig. 1), strain BUT-8^T formed a subclude with *L. ruishenii*, *L. daejeonensis*, *Lysobacter niastensis*, Lysobacter soli, Lysobacter panacisoli *and Lysobacter enzymogenes*, and clustered most closely with *L. ruishenii and L. daejeonensis* (with bootstrap confidence levels of 99 % and 77 %, respectively). Phylogenetic trees inferred by the maximum-parsimony and maximum-likelihood methods showed similar relationships to those indicated by the neighbour-joining method (Figs S2 and S3).

For determination of fatty acid profiles, strain BUT-8^T, *L. daejeonensis* KACC 11406^T and *L. ruishenii* DSM 22393^T were streaked on TSA plates, and cells were harvested from the third quadrant of the quadrant-streaked plate. The fatty acid methyl esters were obtained from cells by saponification, methylation and extraction, and separated in a gas chromatograph (Agilent 6890N). Peaks were automatically integrated and fatty acid names and percentages were determined using the Sherlock Microbial Identification System version 6.0B with the TSBA6 library (MIDI; Sasser, 1990). For determination of polar lipids and quinones, cells growing exponentially in TSB were harvested by centrifugation, washed with distilled water and freeze-dried. Analyses of polar lipids were carried out by the Identification Service, DSMZ, Braunschweig, Germany,

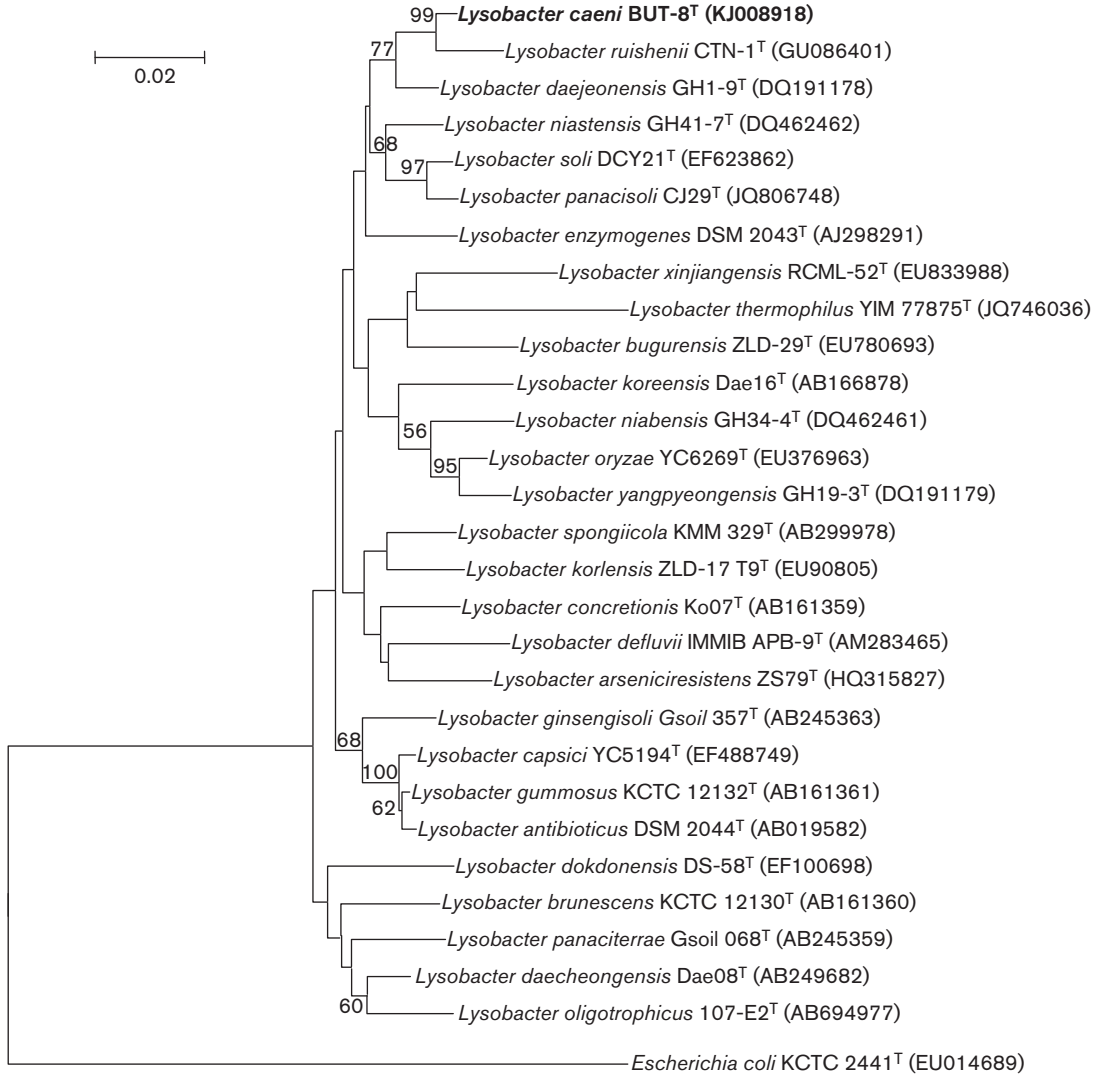

Fig. 1. Phylogenetic tree, reconstructed by the neighbour-joining method, based on the 16S rRNA gene sequences of strain BUT-8^T and the type strains of recognized species of the genus *Lysobacter*. Bootstrap percentages (based on 1000 replications) above 50 % are shown at the nodes. The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. *Escherichia coli* KCTC 2441^T (EU014689) was used as the outgroup. Bar, 0.02 substitutions per nucleotide position. All of the bacteria were standing in the nomenclature on the LPSN bacterio.net website except *Lysobacter daecheongensis* Dae08^T (AB249682) (Ten *et al.*, 2008).

according to methods previously reported (Bligh & Dyer, 1959; Tindall *et al.*, 2007). Quinones were extracted using the method of Collins *et al.* (1977) and analysed by HPLC. The G+C content of the genomic DNA was determined by thermal denaturation (Mandel & Marmur, 1968).

The fatty acid compositions of strain BUT-8^T, *L. daejeonensis* KACC 11406^T and *L. ruishenii* DSM 22393^T are shown in Table 2. The major fatty acids of strain BUT-8^T were iso-$C_{11:0}$ (5.24 %), iso-$C_{15:0}$ (31.66 %), iso-$C_{16:0}$ (21.80 %), iso-$C_{17:0}$ (7.68 %), summed feature 9 (comprising iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl, 14.26 %) and iso-$C_{11:0}$ 3OH (5.72 %). This fatty acid profile was similar to that of other species of the genus *Lysobacter*. However, some qualitative and quantitative differences in the proportions of fatty acids could be observed between the isolate and the reference strains. Compared with *L. ruishenii* DSM 22393^T, strain BUT-8^T had comparatively high levels of iso-$C_{14:0}$ and iso-$C_{16:0}$, and low levels of iso-$C_{11:0}$, iso-$C_{11:0}$ 3OH, iso-$C_{17:0}$ and summed feature 9. Whereas, compared with *L. daejeonensis* KACC 11406^T, strain BUT-8^T had comparatively high levels of $C_{16:0}$ and low levels of iso-$C_{15:0}$. The major respiratory quinone of strain BUT-8^T was ubiquinone-8 (Fig. S4); this is a characteristic feature of the genus *Lysobacter* (Bae *et al.*, 2005). In the polar lipid profile, diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were the major compounds detected. One aminolipid (AL), one phosphoaminolipid and one phospholipid were present in moderate amounts (Fig. S5). DPG, PE and PG are the major common polar lipids present in members of the genus *Lysobacter* (Wang *et al.*, 2011; Luo *et al.*, 2012). However, AL has not been previously reported to be found in species of the genus *Lysobacter*. Phosphatidyl-*N*-methylethanolamine, which is present in many species of the genus *Lysobacter* (Park *et al.*, 2008; Aslam *et al.*, 2009; Srinivasan *et al.*, 2010), was not detected in strain BUT-8^T. The G+C content of strain BUT-8^T was 70.6 mol%. This value is within the range (65.4–70.1 mol%) reported for the genus *Lysobacter* (Christensen & Cook, 1978; Aslam *et al.*, 2009).

The taxonomic relationships between strain BUT-8^T and *L. daejeonensis* KACC 11406^T and *L. ruishenii* DSM 22393^T were further examined using DNA–DNA hybridization (DDH). Total genomic DNA of the three strains was extracted and purified, and DDH was performed using photobiotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989). Hybridizations were repeated three times and the means of the resulting values were determined, and reciprocal experiments were performed. Strain BUT-8^T exhibited relatively low levels of DNA–DNA relatedness with respect to *L. daejeonensis* KACC 11406^T (40.2±5.6 %; reciprocal, 53.5±3.8 %), and *L. ruishenii* DSM 22393^T (57.8±4.2 %; reciprocal, 44.2±5.1 %). The hybridization values were below 70 %, as recommended for the delineation of species (Wayne *et al.*, 1987). Therefore, the phylogenetic distinctiveness, DNA–DNA relatedness data and the differential phenotypic properties are sufficient to show that strain BUT-8^T is distinct to recognized species of the genus *Lysobacter*. On the basis of the data presented, strain BUT-8^T represents a novel species within the genus *Lysobacter*, for which the name *Lysobacter caeni*s sp. nov. is proposed.

Table 2. Fatty acid compositions (as percentages of totals) of strain BUT-8^T and two type strains of species of the genus *Lysobacter*

Strains: 1, BUT-8^T; 2, *L. ruishenii* DSM 22393^T, 3, *L. daejeonensis* KACC 11406^T. All data are from this study. −, Not detected.

Fatty acid	1	2	3
Saturated			
$C_{10:0}$	0.14	0.19	0.05
$C_{14:0}$	0.29	0.41	0.35
$C_{16:0}$	4.84	5.57	2.54
$C_{17:0}$	0.16	0.18	0.06
$C_{18:0}$	−	0.08	−
Unsaturated			
$C_{16:1}\omega7c$ alcohol	−	0.07	−
Branched-chain			
iso-$C_{10:0}$	0.30	0.18	0.16
iso-$C_{11:0}$	5.24	9.32	4.73
anteiso-$C_{11:0}$	0.07	0.06	0.03
iso-$C_{12:0}$	0.30	0.27	0.24
iso-$C_{13:0}$	0.16	0.24	0.18
iso-$C_{14:0}$	2.07	0.48	1.52
iso-$C_{15:0}$	31.66	28.43	40.03
iso-$C_{15:1}$ F	0.88	0.72	1.13
anteiso-$C_{15:0}$	0.88	0.48	0.34
anteiso-$C_{15:1}$ A	−	−	0.04
iso-$C_{16:0}$	21.80	9.16	19.97
iso-$C_{16:1}$ H	0.26	0.09	0.33
$C_{16:0}$ N alcohol	−	0.20	0.15
iso-$C_{17:0}$	7.68	15.23	8.57
anteiso-$C_{17:0}$	0.38	0.35	0.14
iso-$C_{18:0}$	0.35	0.27	0.19
iso-$C_{19:0}$	−	0.06	−
iso-$C_{11:0}$ 3OH	5.72	7.86	4.69
iso-$C_{12:0}$ 3OH	0.17	0.08	0.10
Hydroxy			
$C_{10:0}$ 3OH	0.10	−	0.04
$C_{11:0}$ 2OH	−	0.05	0.07
Cyclo			
$C_{17:0cyclo}$	0.36	0.33	0.09
Summed features*			
3	1.74	1.61	0.51
4	0.12	0.12	−
8	0.08	0.15	0.02
9	14.26	17.64	13.73

*Summed features are composed of fatty acids that could not be separated by the MIDI system. Summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 4 comprises iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B; summed feature 8 comprises $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; summed feature 9 comprises iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl.

Description of *Lysobacter caeni* sp. nov.

Lysobacter caeni (cae'ni. L. gen. n. *caeni* of sludge).

Cells are Gram-stain-negative, aerobic, non-spore-forming, non-motile and long rods, 0.42–0.57 μm × 1.76–2.0 μm. Colonies grown on TSA plates are convex, circular, smooth, non-transparent and yellow-green after 3 days of incubation at 30 °C. Grows at 15–37 °C (optimum 28–30 °C), pH 6.0–9.0 (optimum pH 7.0), and NaCl concentrations of 0–1 % (w/v, optimum 0.5 %). Positive for oxidase, catalase and nitrate reduction, but negative for acid production from glucose and indole. Hydrolyses aesculin, casein, gelatin and urea, but not adenine, cellulose, guanine, starch or Tween 20. Utilizes acetate, L-alanine, glycogen, 3-hydroxybutyric acid, malate, mannose, L-proline, D-ribose and sucrose, but not *N*-acetylglucosamine, D-glucose, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, inositol, maltose, potassium 5-ketogluconate, L-serine, suberic acid or valerate. Positive for acidphosphatase, alkaline phosphatase, esterase (C-4), esterase lipase (C-8), α-glucosidase, leucine aryl amidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine aryl amidase, but negative for *N*-acetylglucosaminidase, α-chymotrypsin, cystine aryl amidase, α-fucosidase, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase, lipase (C-14) or α-mannosidase. The major respiratory quinone is ubiquinone-8, and the major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol with one aminolipid, one phosphoaminolipid and one phospholipid also present in moderate amounts. The main cellular fatty acids (>5 %) are iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{11:0}$, iso-$C_{17:0}$, iso-$C_{11:0}$ 3OH, summed feature 9 (comprising iso-$C_{17:1}\omega9c$ and/or $C_{16:0}$ 10-methyl).

The type strain, BUT-8^T (=CCTCC AB 2013087^T=KACC 17141^T), was isolated from the activated sludge of a herbicide-manufacturing wastewater treatment facility in Kunshan city, Jiangsu Province, PR China. The genomic DNA G+C content of the type strain is 70.6 mol%.

Acknowledgements

This work was supported by the National Science and Technology support plan (2013AA102804), the National Natural Science Foundation of China (grant no. 31270157) and Program for New Century Excellent Talents in University (NCET-13-0861).

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