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Lysobacter spongiae sp. nov., isolated from spongin

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A Gram-negative, motile, aerobic and rod-shaped bacterial strain designated 119BY6-57^T was isolated from spongin. The taxonomic position of the novel isolate was confirmed using the polyphasic approach. Strain 119BY6-57^T grew well at 25–30°C on marine agar. On the basis of 16S rRNA gene sequence similarity, strain 119BY6-57^T belongs to the family *Xanthomonadaceae* and is related to *Lysobacter aestuarii* S2-C^T (99.8% sequence similarity), *L. maris* KMU-14^T (97.5%), and *L. daejeonensis* GH1-9^T (97.3%). Lower sequence similarities (97.0%) were found with all of the other recognized members of the genus *Lysobacter*. The G + C content of the genomic DNA was 69.9 mol%. The major respiratory quinone was Q-8 and the major fatty acids were $C_{16:0}$ iso, $C_{15:0}$ iso, summed feature 9 (comprising $C_{17:1}$ iso *ω9c* and/or $C_{16:0}$ 10-methyl), summed feature 3 (comprising $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*), and $C_{11:0}$ iso 3-OH. The polar lipids were phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol, three unidentified phospholipids, and an unidentified polar lipid. DNA-DNA relatedness values between strain 119BY6-57^T and its closest phylogenetically neighbors were below 48.0 ± 2.1%. Based on genotypic and phenotypic characteristics, it is concluded that strain 119BY6-57^T is a new member within the genus *Lysobacter*, for which the name *Lysobacter spongiae* sp. nov. is proposed. The type strain is 119BY6-57^T (= KACC 19276^T = LMG 30077^T).

Keywords: *Lysobacter spongiae*, 16S rRNA gene, polyphasic taxonomy

Introduction

The genus *Lysobacter* was initially proposed by Christensen and Cook, and the description was amended by Park *et al.* (2008). Members of the genus *Lysobacter* are characterized as aerobic, Gram-negative, non-fruiting, yellow-brownish or pink in color, and with colonies that are mucoid and cream-like.

Ubiquinone-8 (Q-8) is the main respiratory quinone of all species in the genus, and iso-branched fatty acids usually predominate. G + C content of DNA is 61.7–70.7 mol% (Park *et al.*, 2008; Wang *et al.*, 2009). Phylogenetic analyses have revealed that the genus *Lysobacter* is closely related with the genera *Xanthomonas* and *Pseudomonas* within the family *Xanthomonadaceae*. The genus *Lysobacter* contains more than 40 recognized species (Euzéby, 1997) isolated from various environments. These include the recently described species *Lysobacter aestuarii* (Jeong *et al.*, 2016), *L. hankyongensis*, and *L. sediminicola* (Siddiqi and Im, 2016a), *L. maris* (Yoon, 2016), *L. pocheonensis* (Siddiqi and Im, 2016b), *L. terricola* (Kim *et al.*, 2016), and *L. caeni* (Ye *et al.*, 2015).

Materials and Methods

Strain isolation

Strain 119BY6-57^T was isolated from spongin on Jeju Island, Republic of Korea (33°14′11.6″ N, 126°35′59.6″ E). The spongin samples were dissolved in sterilized distilled water (DW). Each solution was serially diluted and aliquots were spread on marine agar medium (BD) and incubated at 30°C for 3 days. The colonies were purified by subculture on fresh marine agar plates at 30°C and preserved in marine broth supplemented with 20% glycerol (w/v) at -80°C.

Phenotypic, biochemical, and hydrolysis characteristics of strain 119BY6-57^T

As previously described by Buck (1982), Gram-staining was carried out. Cell shape and size was determined by transmission electron microscopy using a JEN1010 microscope (JEOL) operating at an accelerating voltage of 80 kV. Cell motility was assessed by light microscopy using an Optiphot-2 microscope (Nikon) at 1,000 × as previously described (Perry, 1973) after growth of cells on marine agar for 3 days at 30°C. Cells grown on marine agar for 1 day were used for determination of catalase and oxidase activity (Cappuccino and Sherman, 2002), and for phenotypic and biochemical tests. Anaerobic growth was tested on marine agar (Difco) in a Pouch System Anaerobe Gas Generator (BD) for up to 7 days at 30°C. Biochemical tests used API 20NE, API ID 32GN, and API ZYM strips according to the manufacturer's instructions (bioMérieux). Determination of the hydrolysis of Tween-80, DNA, starch, casein, and carboxymethyl-cellulose were performed as outlined by Atlas (1993) and Ten *et al.* (2004). After 5 days of incubation at 30°C, the results were evaluated.

Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, 42, and 45°C), pH (pH 4.5–10.0 in increments of 0.5), and

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The GenBank accession number for the 16S rRNA gene sequence of strain 119BY6-57^T is KY451771.

agar media (nutrient agar, trypticase soy agar, LB agar, and MacConkey agar; all from Difco) were assessed on marine medium at 30°C after 5 days of incubation. Salt tolerance was also evaluated using marine broth supplemented with 1–10% (w/v) NaCl (in increments of 1%) at 30°C after 5 days of incubation.

Phylogenetic analysis

The DNA of the novel isolate was extracted using a genomic DNA extraction kit (Macrogen) and subjected to PCR amplification of the 16S rRNA gene. The gene was sequenced as previously described (Kim *et al.*, 2015). The nearly full-length of the 16S rRNA gene sequence was assembled using SeqMan software (DNASTAR). Similarly, the 16S rRNA gene sequences of related reference strains were obtained from the EzBiocloud [http://www.ezbiocloud.net] server (Yoon *et al.*, 2017) and GenBank database. The Clustal_X program was used for multiple sequence alignments (Thompson *et al.*, 1997) and the gaps were edited using the BioEdit program (Hall, 1999).

Fig. 1. Transmission electron micrograph of strain 119BY6-57^T. Bar represents 0.5 μm.

Similarly, the Kimura two-parameter model (KM2; Kimura, 1983) was used to calculate the evolutionary distances. Phylogenetic trees involving maximum-parsimony (Fitch, 1971)

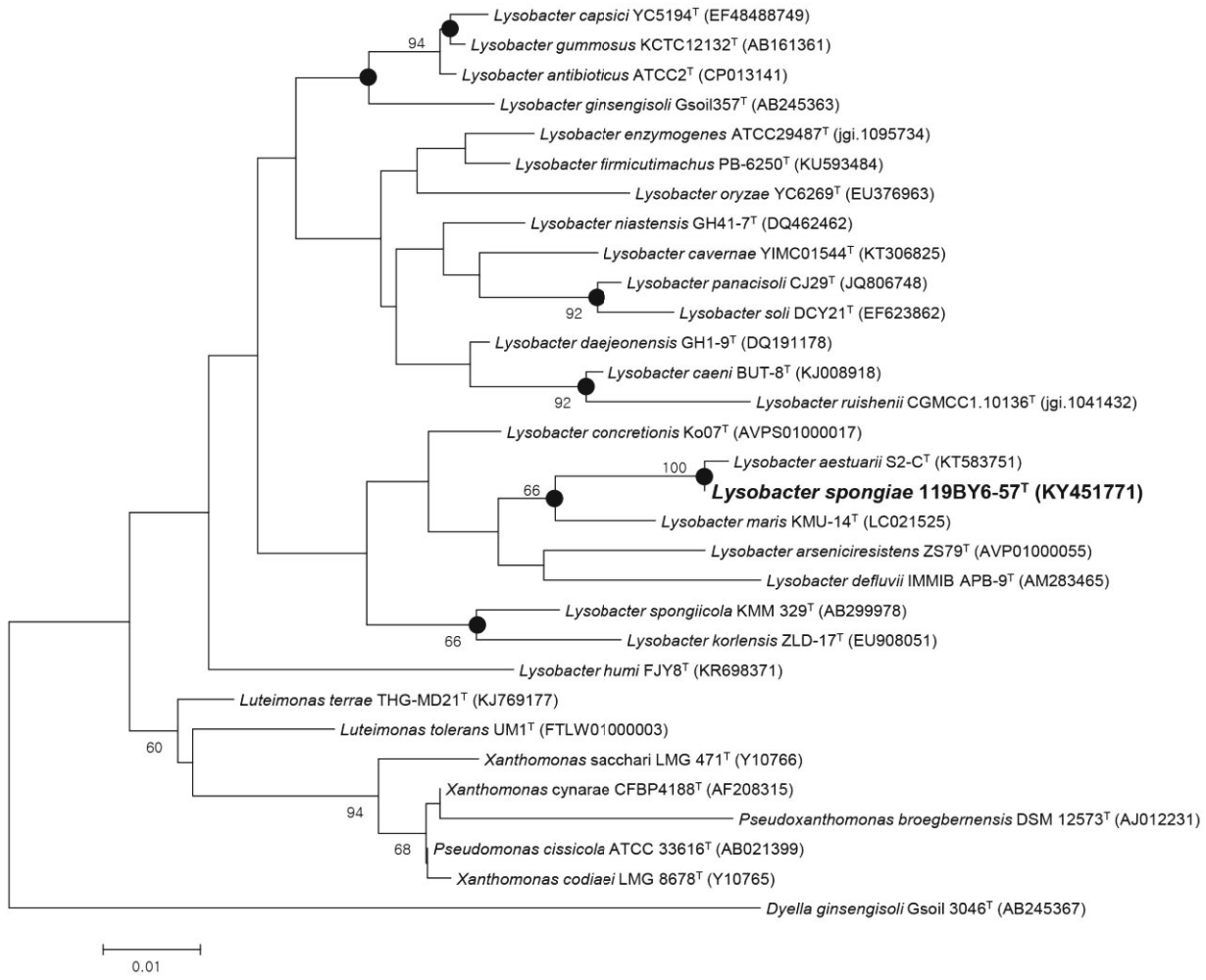

Fig. 2. Phylogenetic relationship between strain 119BY6-57^T and other related species of the genus *Lysobacter* of the family *Xanthomonadaceae*. The tree was constructed using the maximum-likelihood method based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1,000 replications) greater than 60% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with neighbor-joining and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

Table 1. Differentiating characteristics of *Lysobacter spongiae* 119BY6-57^T and the type strains of related *Lysobacter* species

Strains: 1, *L. spongiae* 119BY6-57^T; 2, *L. caeni* KACC 17141^T; 3, *L. ruishenii* KCTC 23715^T; 4, *L. daejeonensis* KACC 11406^T; 5, *L. concretionis* KACC 11484^T; 6, *L. arseniciresistens* KCTC 23365^T; 7, *L. aestuarii* KACC 18502^T; 8, *L. maris* KCTC 42381^T. In API kit system (ZYM, 20NE, and 32GN), all strains were positive for urease, alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, acid phosphatase, naphthalol-AS-BI-phosphohydrolase, protease and acetate. Negative for indole production, arginine dihydrolase, phenyl-acetate, cysteine arylamidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, D-melibiose, citrate, and itaconate. +, positive; w, weak positive; -, negative.

Characteristics	1	2	3	4	5	6	7	8
Temperature (Optimum) (°C)	10–37 (20–30)	15–37 (28–30) [a]	15–37 (28–30) [b]	10–37 (28) [c]	25–30 (28) [d]	4–37 (28) [e]	15–40 (30) [f]	15–37 (28) [g]
pH range (Optimum)	6–10 (6)	6.0–9.0 (7.0) [a]	6.0–9.0 (7.0–7.5) [b]	6.0–8.0 (7.0) [c]	6.8–7.5 (7.0) [d]	5–9 (7) [e]	5.5–9.0 (6.5-7.5) [f]	6.0–9.0 (7.0) [g]
NaCl range (Optimum) (%)	0–9 (1–2)	0–1 (0.5) [a]	0–1 (0.5) [b]	0–3 (0.5) [c]	0–3 (0) [d]	0–4 (0) [e]	0–7 (0–2) [f]	0–5 (0–3) [g]
Hydrolysis of:								
DNA	-	-	-	+	-	+	-	-
Casein	+	+	+	+	+	-	+	+
API 20 NE & ID32 GN results								
Nitrate reduction	+	+	+	-	-	-	-	-
Glucose Acidification	-	-	-	-	-	+	+	-
Urease	-	-	-	-	+	-	-	-
D-Glucose	+	-	+	+	+	-	-	+
L-Arabinose	+	-	-	-	+	-	-	-
D-Mannose	+	-	-	-	w	-	-	-
D-Mannitol	-	-	-	-	+	-	-	-
N-Acetyl-glucosamine	+	-	+	-	+	-	+	-
D-Maltose	+	+	-	+	+	-	+	+
Gluconate	w	w	w	-	w	+	+	+
Caprate	+	-	-	-	-	-	-	-
Adipate	-	w	-	w	-	-	-	-
Malate	+	-	+	-	+	-	-	-
Salicin	-	-	-	+	-	-	+	-
L-Fucose	+	-	+	-	-	+	+	+
D-Sorbitol	+	-	+	-	-	+	+	+
Propionate	+	+	+	+	-	+	+	+
Valerate	-	+	+	+	+	+	+	+
L-Histidine	+	-	-	-	+	+	+	+
2-Ketogluconate	+	-	+	-	-	+	-	+
3-Hydroxy-butyrate	-	+	+	+	+	+	+	-
4-hydroxy-benzoate	+	+	+	-	-	+	+	+
L-Proline	+	+	-	-	+	-	+	+
L-Rhamnose	+	-	-	-	-	-	-	-
D-Ribose	+	-	-	+	-	-	-	-
Inositol	+	-	+	-	-	-	+	+
D-Sucrose	+	-	-	-	+	+	+	-
Suberate	-	-	-	-	-	+	-	-
Malonate	-	-	-	-	-	+	-	-
DL-Lactate	+	-	-	-	-	+	-	+
L-Alanine	+	+	-	+	-	+	-	+
5-Ketogluconate	-	-	+	-	-	+	+	+
Glycogen	-	+	+	+	+	+	+	-
3-Hydroxy-benzoate	-	-	+	-	-	+	-	-
L-Serine	+	+	-	+	-	-	-	-
API ZYM results								
Lipase (C14)	-	-	-	-	-	+	-	-
Valine arylamidase	-	+	-	+	-	+	w	-
Trypsin	+	+	+	-	+	+	-	+
α-Chymotrypsin	+	-	-	w	-	+	+	+
α-Galactosidase	-	-	-	-	+	-	-	-
α-Glucosidase	+	+	-	-	+	+	-	+
N-Acetyl-β-glucosaminidase	+	-	-	w	-	-	+	-
Isolation source	Spongin	Sludge	Soil	Soil	Sludge	Soil	Sediment	Seawater
G+C content (mol %)	69.9	70.6[a]	67.1[b]	61.7[c]	63.8[d]	70.7[e]	63.8[f]	64.9[g]

[a] *L. caeni* KACC 17141^T (Ye *et al.*, 2015)
[b] *L. ruishenii* KCTC 23715^T (Wang *et al.*, 2011)
[c] *L. daejeonensis* KACC 11406^T (Weon *et al.*, 2006)
[d] *L. concretionis* KACC 11484^T (Bae *et al.*, 2005)
[e] *L. arseniciresistens* KCTC 23365^T (Luo *et al.*, 2012)
[f] *L. aestuarii* KACC 18502^T (Jeong *et al.*, 2016)
[g] *L. maris* KCTC 42381^T (Yoon, 2016)

neighbor-joining (Saitou and Nei, 1987), and maximum likelihood were constructed using the MEGA 6 Program (Tamura *et al.*, 2013) with the bootstrap values of 1,000 replicates (Felsenstein, 1985).

Chemotaxonomy analysis of the novel isolate

Polar lipids, quinone, and cellular fatty acids : Polar lipid content of strain 119BY6-57^T was determined as previously described (Minnikin *et al.*, 1984). Cell biomass used to analyze isoprenoid quinones was obtained from cultures grown on marine agar for 3 days at 30°C. The quinones were extracted with chloroform/methanol [C/M at a 2:1 (v/v) ratio], evaporated under a vacuum, and re-extracted in *n*-hexane-water (1:1, v/v). The purified quinone was analyzed using a reverse-phase HPLC system (Younglin) as previously described (Hiraishi *et al.*, 1996). The cellular fatty acid profiles of strain 119BY6-57^T and its closest reference strains were also determined following growth for 60 h at 30°C on marine agar. The fatty acids were extracted according to the protocol of the Sherlock Microbial Identification (MIDI) system used. Fatty acid methyl esters were examined by gas chromatography using a model 6890 apparatus (Hewlett Packard) and the TSBA library (version 6.1) and MIDI system (Sasser, 1990).

DNA G + C content

The DNA G + C content of strain 119BY6-57^T was determined from DNA extracted and purified as previously described (Moore and Dowhan, 1995), and degraded enzymatically into the constituent nucleosides. The G + C content was determined as described by Mesbah *et al.* (1989), using reverse-phase HPLC.

DNA-DNA hybridization

DNA hybridization experiments were performed between strain 119BY6-57^T and two reference strains (*Lysobacter aestuarii* KACC 18502^T, *Lysobacter maris* KCTC 42381^T) using photobiotin-labeled DNA probes and micro-dilution wells (Ezaki *et al.*, 1989). The hybridization experiments were completed at 52.9°C with five replications. The highest and lowest values were omitted for each sample, and the means of the remaining three values were used as the DNA-DNA relatedness values.

Results and Discussion

Physiological characteristics

Cells of strain 119BY6-57^T were Gram-negative, aerobic, and rod shaped (Fig. 1). They were oxidase and catalase positive. Colonies on marine agar plates that developed during 3 days of incubation at 30°C were yellow-orange, smooth, circular, and 0.5–2 mm in diameter. On marine agar, 119BY6-57^T grew at 10–37°C, but not at 8 and 40°C. The isolate grew on nutrient agar, TSA, R2A agar, LB agar, and MacConkey agar. The phenotypic and chemotaxonomic characteristics that differentiated strain 119BY6-57^T from closely related *Lysobacter* species are listed in Table 1.

Phylogenetic tree analysis

The 16S rRNA gene sequence of strain 119BY6-57^T consisted of 1,405 bp. The sequence has been deposited in the GenBank database under accession number KY451771. The sequence similarity determined using the EzBiocloud server indicated that strain 119BY6-57^T shared less than 99.8% 16S rRNA gene sequence similarity with all other related taxa of the genus *Lysobacter*.

The highest sequence similarity of the novel isolate was found with *L. aestuarii* S2-C^T (99.8%), *L. maris* KMU-14^T (97.5%), and *L. daejeonensis* GH1-9^T (97.3%).

Based on the phylogenetic trees analysis, strain 119BY6-57^T clearly constituted a separate branch within the genus *Lysobacter* and formed a group with *L. aestuarii* S2-C^T, *L. maris* KMU-14^T, *L. arseniciresistens* ZS79^T, *L. concretionis* Ko07^T, *L. ruishenii* CGMCC1.10136^T, *L. caeni* BUT-8^T, and *L. daejeonensis* GH1-9^T (Fig. 2). Thus, based on the phylogenetic tree analysis, the aforementioned seven strains were selected as closest reference strains for the comparative analysis with strain 119BY6-57^T.

Fatty acids, polar lipids, quinone, and polyamine analyses

The main detected respiratory quinone of strain 119BY6-57^T was Q-8, which was similar to the other members of the genus *Lysobacter*. The cellular fatty acids of strain 119BY6-57^T and its closest reference strains are shown in Table 2. The major fatty acids in strain 119BY6-57^T and its three reference strains were summed feature 9 (comprising iso-$C_{17:1}$ ω9*c* and/or $C_{16:0}$ 10-methyl), iso-$C_{15:0}$, and iso-$C_{16:0}$. Some qualitative and quantitative differences in fatty acid content were observed between strain 119BY6-57^T and its phylogenetically closest relatives. The main polar lipids were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG). Minor polar lipids comprised three unidentified phospholipids (PLs) and one unidentified polar lipid (Fig. 3).

DNA G + C content and DNA-DNA hybridization analysis

The G + C content of strain 119BY6-57^T was 69.9 mol%, which was similar to previously described species of genus *Lysobacter* (Table 1). The DNA hybridization relatedness values between strain 119BY6-57^T, *L. aestuarii* S2-C^T, and *L. maris* KMU-14^T were 48.0 ± 2.1% and 20.4 ± 1.6%, respectively. The results indicate that the DNA-DNA relatedness values were lower than the threshold value of 70% for the delineation of genospecies (Wayne *et al.*, 1987). Therefore, it is clear that strain 119BY6-57^T should be considered a new species within the genus *Lysobacter*.

Taxonomic conclusion

These pilot data indicate that strain 119BY6-57^T shares numerous features in common with the members of the genus *Lysobacter*, which include Q-8 as the major quinone; DPG, PG, and PE as the major lipids; and summed feature 9, iso-$C_{16:0}$, and iso-$C_{15:0}$ as major fatty acids. Even though the phylogenetic distance of the 16S rRNA gene sequence is close, the DNA hybridization analysis, chemotaxonomic, and phenotypic characteristics differentiate the novel isolate from

Table 2. Cellular fatty acid profiles of strain 119BY6-57^T and recognized species of genus *Lysobacter*

Strains: 1, *L. spongiae* 119BY6-57^T; 2, *L. caeni* KACC 17141^T; 3, *L. ruishenii* KCTC 23715^T; 4, *L. daejeonensis* KACC 11406^T; 5, *L. concretionis* KACC 11484^T; 6, *L. arseniciresistens* KCTC 23365^T; 7, *L. aestuarii* KACC 18502^T; 8, *L. maris* KCTC 42381^T. All cells were cultured on marine agar for 60 h at 30°C. TR, trace amount (< 0.5%).

	1	2	3	4	5	6	7	8
Fatty acid								
Saturated								
$C_{10:0}$	TR	TR	0.6	TR	0.6	1.1	TR	1.1
$C_{12:0}$	0.5	0.9	0.7	1.3	TR	0.7	TR	TR
$C_{14:0}$	TR	0.6	1.2	1.2	1.4	1.9	TR	TR
$C_{16:0}$	3.5	8.7	5.2	4.6	8.4	10.8	TR	4.3
$C_{18:0}$	TR	TR	TR	0.9	TR	TR	TR	TR
Branched-chain fatty acid								
$C_{10:0}$ iso	0.5	TR	TR	TR	0.5	TR	TR	TR
$C_{11:0}$ iso	4.7	4.5	3.6	3.7	2.9	3.0	11.6	7.5
$C_{11:0}$ iso 3OH	6.1	6.0	5.4	8.8	4.2	4.4	9.3	5.1
$C_{12:0}$ iso	0.6	TR	0.7	0.5	0.9	0.7	1.3	1.2
$C_{14:0}$ iso	3.6	0.6	9.7	1.3	6.4	3.6	5.3	5.1
$C_{15:0}$ iso	16.4	24.0	18.2	30.8	13.3	14.2	19.0	21.2
$C_{15:1}$ iso F	TR	0.8	3.2	1.3	2.4	1.1	TR	2.1
$C_{16:0}$ iso	31.8	12.6	29.8	16.5	34.6	28.1	22.4	22.8
$C_{16:0}$ N alcohol	TR	TR	TR	TR	0.5	TR	TR	TR
$C_{16:1}$ iso H	0.9	TR	0.8	TR	1.6	0.6	TR	TR
$C_{17:0}$ iso	2.5	12.6	1.7	9.4	1.7	2.6	1.9	2.0
$C_{18:0}$ iso	1.5	TR	TR	TR	TR	TR	TR	TR
$C_{15:0}$ anteiso	1.1	1.6	2.3	1.1	2.6	3.9	1.1	2.4
$C_{17:0}$ anteiso	TR	1.0	TR	TR	TR	0.9	TR	TR
Hydroxy fatty acids								
$C_{10:0}$ 3OH	TR	TR	TR	TR	TR	0.9	TR	TR
CYCLO								
$C_{17:0}$ cyclo	1.3	TR	0.7	1.0	1.0	0.8	TR	1.2
Summed feature								
3; $C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*	6.9	3.0	3.9	1.5	4.9	11.0	4.6	8.5
8; $C_{18:1}$ *ω7c*/$C_{18:1}$ *ω6c*	1.0	TR	TR	TR	TR	TR	TR	TR
9; $C_{17:1}$ iso *ω9c*/$C_{16:0}$ 10-methyl	13.1	19.9	9.6	13.8	9.5	7.2	14.5	8.6

Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system.

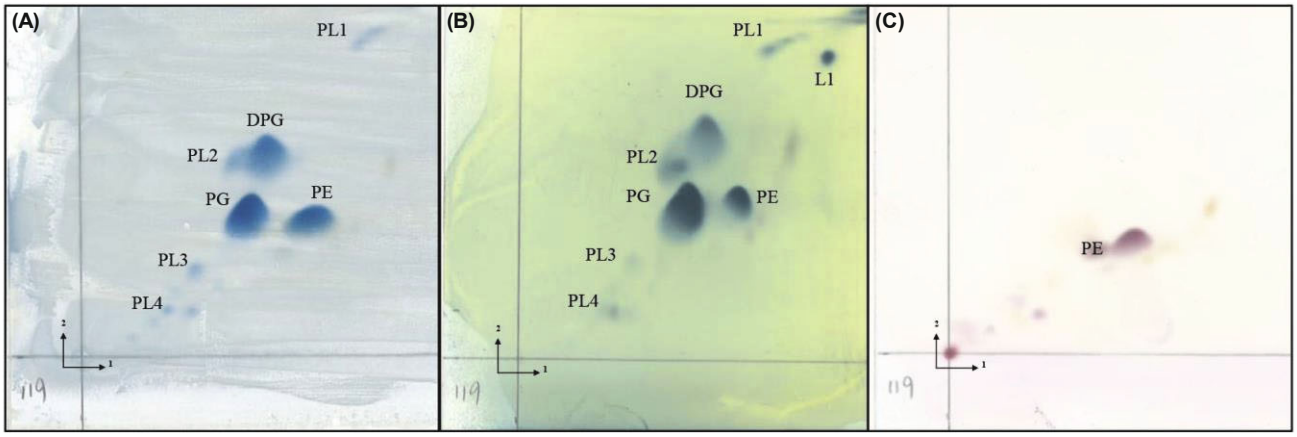

Fig. 3. Two-dimensional thin-layer chromatogram of polar lipids of strain 119BY6-57^T. Chloroform/methanol/water (65:25:4, by vol.) was used in the first direction, followed by chloroform/acetic acid/methanol/water (80:15:12:4, by vol.) in the second direction. The following spray reagents were used for detection: (A) molybdenum blue (Sigma) (for phospholipids); (B) 5% ethanolic molybdophosphoric acid (Sigma) (for total lipids); (C) ninhydrin (Sigma) (for aminolipids). Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL, unidentified phospholipids (PL1-PL3); L, unidentified polar lipids (L1, L2).

previously recognized species of the genus *Lysobacter* (Tables 1 and 2). Therefore, strain 119BY6-57^T should be allocated to the genus *Lysobacter* with the proposed name of *Lysobacter spongiae* sp. nov.

Description of *Lysobacter spongiae* sp. nov.

Lysobacter spongiae (spon.gi'ae. L. gen. n. spongiae of a sponge, the source of the type strain)

Cells are Gram-negative, strictly aerobic, motile by gliding, and rod-shaped (0.3–0.6 μm in diameter and 1.5–3.0 μm in length) after culture on marine agar for 3 days at 30°C. Colonies grown on marine agar medium are smooth, convex, circular, yellow-orange in color, and 0.5–2.0 mm in diameter after 3 days of incubation at 30°C on marine agar. Growth also occurs on marine agar, TSA, R2A, LB, nutrient agar, and MacConkey agar. Growth occurs on marine agar at 10–37°C at pH 6.0–10.0 and 1–9% (w/v) NaCl. Optimum growth occurs at 30°C and pH 6.0 and 1–2% NaCl (w/v). Positive for catalase and oxidase, and hydrolyzes casein but does hydrolyze carboxymethyl-cellulose, starch, and DNA. Carbon assimilation (API ID 32 GN, API 20 NE) and enzyme activities (API ZYM) are summarized in Table 1. The predominant respiratory quinone is Q-8, and $C_{16:0}$ iso, $C_{15:0}$ iso, and summed feature 9 (comprising $C_{17:1}$ iso *ω9c*, and/or $C_{16:0}$ 10-methyl) are the major cellular fatty acids (> 6.5%). The major polar lipids are DPG, PG, and PE. The G + C content of the genomic DNA is 69.9 mol%.

The type strain, 119BY6-57^T (= KACC 19276^T = LMG 30077^T) was isolated from spongin on Jeju Island, Republic of Korea.

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