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Lysobacter hymeniacidonis sp. nov., Isolated from a Crude Oil-Contaminated Marine Sponge

XIN Yanjuan[1)], QU Junge[2)], XU Junyi[3)], WU Peichun[1)], CAO Xupeng[1)], and XUE Song[1), *]

1) *Marine Bioproducts Engineering Group*, *Dalian Institute of Chemical Physics*, *Chinese Academy of Sciences*, *Dalian* 116023, *P. R. China*
2) *Department of Biology and Pharmacy*, *Zhejiang Pharmaceutical College*, *Ningbo* 315100, *P. R. China*
3) *Liaoning Entry-Exit Inspection and Quarantine Bureau*, *Dalian* 116001, *P. R. China*

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Abstract An aerobic, Gram-negative bacterium, strain 2-5^T, was isolated from a crude oil-contaminated marine sponge collected near Dalian Bay, China, and subjected to a polyphasic taxonomic investigation. Cells of strain 2-5^T were non-spore forming, non-motile, rods 0.2–0.3 μm wide and 1.1–1.2μm long. Strain 2-5^T grew well on nutrient agar, TSA, R2A agar and LB agar. Colonies of strain 2-5^T on LB agar were circular, smooth with entire margins, non-transparent and pale yellow after 3 d of incubation at 30℃. Growth of strain 2-5^T occurred in LN medium with 0–6% NaCl; no growth occurred in the presence of 8.0% NaCl. Strain 2-5^T grew at 15–42℃ and at pH 6.0–8.0. Comparative 16S rRNA gene sequence analysis showed that strain 2-5^T clustered with the species of the genus *Lysobacter*. Its closet neighbors were the type strains of *Lysobacter concretionis* KCTC 12205^T (97% similarity), *Lysobacter arseniciresistens* ZS79^T (96%), and *Lysobacter defluii* APB-9^T (96%). The value for DNA-DNA relatedness between strain 2-5^T and *L. concretionis* KCTC 12205^T was 23%. Branched fatty acids iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{11:0}$ 3-OH, iso-$C_{17:1}\omega 9c$ and iso-$C_{11:0}$ were found to be predominant. The major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol. Strain 2-5^T had a DNA G+C content of 63.8 mol%. On the basis of the phenotypic, chemotaxonomic, DNA-DNA hybridization and phylogenetic data, strain 2-5^T represents a novel species of the genus *Lysobacter*, for which the name *Lysobacter hymeniacidonis* sp. nov. is proposed. The type strain is 2-5^T (=CGMCC 1.12190^T = JCM 18137^T).

Key words *Lysobacter hymeniacidonis* sp. nov.; taxonomy; phylogenetic analysis

1 Introduction

The genus *Lysobacter* was first described by Christensen and Cook (1978), and the description was emended by Park *et al*. (2008). The genus *Lysobacter*, grouped in the family *Xanthomonadaceae*, is classified in the class *Gammaproteobacteria* (Christensen and Cook, 1978) based on non-fruiting bodies, lack of flagella, gliding nature and high genomic DNA G+C content (typically ranging between 65.4 and 70.1 mol%) (Aslam *et al*., 2009). At the time of writing, the genus *Lysobacter* comprises 27 species with validly published names (http://www. bacterio. cict. fr/). Recently, three novel species of the genus, *Lysobacter arseniciresistens* ZS79^T from iron-mined soil (Luo *et al*., 2012), *Lysobacter oligotrophicus* 107-E2^T from an Antarctic freshwater lake (Fukuda *et al*., 2013) and *Lysobacter panacisoli* CJ29^T from ginsengsoil (Choi *et al*., 2014) have been described. Members of the genus were of great potential for the development of biocontrol agents against plant fungal pathogens (Islam *et al*., 2005; Park *et al*., 2008) and antibiotic bioactivities against human pathogens (Ahmed *et al*., 2003; Hashizume *et al*., 2004). Here we report the characterization of a novel marine bacterium, strain 2-5^T, of the genus *Lysobacter*, which was isolated from a crude oil-contaminated marine sponge.

2 Materials and Methods

2.1 Bacterial Strains

Strain 2-5^T was isolated from a crude oil-contaminated sponge specimen (*Hymeniacidon sinapium*), collected at the inter-tidal beach of Dalian, on the Chinese Yellow Sea, located in northern China (38°52′N 121°41′E). Freshly collected sponge specimens were rinsed five times in sterile seawater to remove unassociated bacteria and then thoroughly homogenized in a sterile mortar. A 10-fold dilution series of sponge homogenate was made and plated on 2216E plates (Difco, USA) in triplicate. After incubating the plates at 28℃ for 7 d, an isolate, designated 2-5^T, was picked and sub-cultured on 2216E plates,

* Corresponding author. Tel: 0086-411-84379069
E-mail: xuesong@dicp.ac.cn

repeating the above steps until a pure culture was obtained. The novel strain was deposited into the CGM CC (China General Microbiological Culture Collection Center) as CGMCC 1.12190^T and the JCM (Japan Collection of Microorganisms) as JCM 18137^T. The reference strain used for the DNA-DNA homology tests was *Lysobacter concretionis* KCTC 12205^T, obtained from the KCTC (Korean Collection for Type Cultures).

2.2 Morphology and Physiological Characteristics

To investigate the morphological and physiological characteristics, cell grown on R2A agar plates at 30℃ for 2 d. Cell morphology and motility were examined using light microscopy (Olympus; ×1000) and transmission electron microscopy (H-7650; Hitachi, Japan) using cells from an exponentially growing culture. The strain Gram straining reaction was carried out according to the classical procedure described by Doetsch (1981). Gliding motility was determined as described by Bowman (2000). The physiological properties of strain 2-5^T were determined by the CGMCC using established procedures described by Gordon *et al*. (1974) and Yokota *et al*. (1993). Catalase activity, oxidase activity, enzyme activity and acid production from different carbohydrates were determined by the CGMCC with Biolog GN2, API 20E, API 20NE kits according to the manufacturer's instructions. The assimilation of single carbon substrates was determined by the CGMCC with Biolog GN2 and API 20NE strips cultured at 28℃ for 24 h. Hydrolysis of casein and chitin was determined using previously described test (Smibert and Krieg, 1994; Brown, 2007). Growth at 4, 10, 15, 25, 30, 37, 42 and 45℃ and at pH 4.0–10.0 (at intervals of 1.0 pH unit) was assessed after 5 d of incubation on 2216E agar. Growth on nutrient agar, trypticase soy agar (TSA; Difco), R2A agar (Difco, USA) and LB agar (Difco, USA) was also evaluated at 28℃. Salt tolerance was tested in LN medium (LB without NaCl) supplemented with 0–10% NaCl after 10 d of incubation.

2.3 G+C Content and Analysis of Cellular Fatty Acids

Cell grown on R2A agar plates at 30℃ for 2 d were used for the analysis of cellular fatty acid and polar lipid. The fatty acids were extracted, methylated and analysed using the standard Sherlock MIDI (Microbial Identification) system (Sasser, 1990; Kämpfer and Kroppenstedt, 1996). Polar lipids were extracted and analyzed as described by Tindall (1990). A 6.75 mL portion of chloroform/methanol/0.3% aqueous NaCl (1: 2: 0.8) was added to 100 mg freeze-dried cell material. The preparation was stirred overnight and cell debris was pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3% aqueous NaCl mixture to a ratio of 1: 1: 0.9 and then dried under nitrogen. The dried polar lipids were resuspended in chloroform/methanol (2: 1) and separated by two-dimensional TLC.

The G+C content of the genomic DNA was determined by the CGMCC by thermal denaturation (Mandel and Marmur, 1968), for which *Escherichia coli* K-12 (CGMCC 1.365) was used as a standard.

2.4 Phylogenetic Analysis and DNA-DNA Hybridization

Genomic DNA from strain 2-5^T was extracted and purified according to standard procedures (Sambrook and Russell, 2001). The 16S rRNA gene cloned into pMD-18T (Takara, Japan) was sequenced using an automated sequencer (Applied Biosystems model 3730). The 16S rRNA gene sequence of strain 2-5^T was compared with known sequences found in the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed with MEGA5 program (Tamura *et al*., 2011) after multiple alignments of the data via CLUSTAL_X program (Thompson *et al*., 1997). A distance matrix method (distance options according to the Kimura two-parameter model), including clustering using the neighbor-joining and maximum-likelihood (Kluge and Farris, 1969) method. In each case, bootstrap values were calculated based on 1000 replications (Felsenstein, 1985). The taxonomic relationship between strain 2-5^T and its phylogenetic relative was further examined using DNA-DNA hybridization. DNA–DNA hybridization values between 2-5^T and *L. concretionis* KCTC 12205^T was performed fluorometrically, according to the method developed by Ezaki *et al*. (1989) using photobiotin-labelled DNA probes and micro dilution wells. Hybridization was conducted in five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the remaining three values were utilized in the calculation of hybridization values.

3 Results and Discussion

3.1 Morphological and Physiological Characteristics

Cells of strain 2-5^T were Gram-negative, non-spore forming, non-motile (but showing gliding activity), aerobic rods 0.2–0.3 μm wide and 1.1–1.2 μm long (Supplementary Fig.S1-2). Strain 2-5^T grew well on nutrient agar, TSA, R2A agar and LB agar. Colonies of strain 2-5^T on LB agar were circular, smooth with entire margins, non-transparent and pale yellow after 3 d of incubation at 30℃. Growth of strain 2-5^T occurred in LN medium with 0–6% NaCl; no growth occurred in the presence of 8.0% NaCl. Strain 2-5^T grew at 15–42℃ (optimal 28–30℃) and at pH 6.0–8.0 (optimum pH 7.0). In the Biolog GN2, API 20E and API 20NE kits, strain 2-5^T assimilated glycogen, *N*-acetyl-D-galactosamine, *N*-acetylglucosamine, ribitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, alpha-D-glucose, M-inositol, D-galactonic acid lactone, D-galacturonic acid, D, L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinamic acid, glucuronamide, L-ala- ninamide, L-alanine, L-alanyl-glycine, L-asparagine, L- aspartic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy-

Table 1 Physiological and biochemical characteristics that differentiate strain 2-5^T from related type strains of the genus *Lysobacter*

Characteristic	1	2	3	4
Gliding motility	+	+	+	+
Growth at 5℃	-	+	+	-
Growth at pH 4	-	-	-	ND
Growth at pH 10	-	-	-	ND
Nitrate reduction	-	-	-	-
Hydrolysis of:				
Aesculin	+	-	-	-
Gelatin	+	+	+	+
Chitin	-	-	ND	-
Starch	-	-	ND	-
Casein	+	+	DN	+
Catalase	+	+	+	+
oxidase	+	+	+	+
β-Galactosidase	-	-	-	-
Salinity range (%)	0–6	0–3	0–4	0–6
Assimilation of:				
D-Glucose	+	-	-	-
L-Arabinose	+	-	-	-
D-Mannose	–	-	-	-
D-Mannitol	+	-	-	+
N-Acetylglucosamine	+	-	-	-
Maltose	+	-	-	+
Trisodium citrate	+	-	-	-
DNA G+C content (mol%)	63.8	63.8	70.0	67.1

Notes: Strains: 1, 2-5^T (data from this study); 2, *L. concretionis* KCTC 12205^T (Bae *et al*., 2005); 3, *L. arseniciresistens* ZS79^T (Lue *et al*., 2012); 4, *L. defluvii* DSM 18482^T (Yassin *et al*., 2007). +, positive; -, negative; ND, no data available.

L-proline. The phenotypic characteristics of strain 2-5^T are summarized in the species description and a comparison of selective characteristics with refe- rence strains are summarized in Table 1.

3.2 G+C content and Analysis of Cellar Fatty Acids

Table 2 Cellular fatty acid compositions (%) of strain 2-5^T and related type strains of the genus *Lysobacter*.

Fatty acid	1	2	3	4
$C_{8:0}$ 3-OH	1.0	-	-	-
iso-$C_{10:0}$	1.3	-	-	-
$C_{10:0}$ 3-OH	1.7	-	-	-
iso-$C_{11:0}$	7.7	10.8	12.6	10.9
iso-$C_{11:0}$ 3-OH	8.4	9.8	12.4	8.5
iso-$C_{14:0}$	1.2	1.4	-	-
iso-$C_{15:0}$	14.4	30.6	28.6	28.8
anteiso-$C_{15:0}$	2.1	1.0	-	-
$C_{16:0}$	5.3	1.9	1.5	-
iso-$C_{16:0}$	15.1	15.5	13.6	27.6
$C_{16:0}$ω7*c*/16:1ω6*c*	6.5	-	-	-
$C_{17:0}$ cyclo	2.3	1.6	-	1.0
iso-$C_{17:0}$	2.5	2.7	4.9	5.6
iso-$C_{17:1}$ω9*c*	9.5	15.4	19.9	9.0
iso-$C_{17:0}$ I/anteiso B	1.2	-	-	-
anteiso-$C_{17:0}$	1.3	-	-	-
$C_{18:1}$ω7*c*	4.0	-	-	-
$C_{18:0}$ 3-OH	1.3	-	-	-
$C_{19:0}$ cyclo ω8*c*	5.2	-	-	-

Notes: Strains: sames as those in Table 1. -, <1% or not detected.

The major cellular fatty acids of strain 2-5^T were iso-$C_{16:0}$ (15.1%), iso-$C_{15:0}$ (14.4%), iso-$C_{17:1}$ω9*c* (9.9%), iso-$C_{11:0}$ 3-OH (8.4%) and iso-$C_{11:0}$ (7.7%) (Table 2). The presence of branched fatty acids, namely iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{17:1}$ω19*c* and iso-$C_{17:0}$, was consistent with the placement of strain 2-5^T within the genus *Lysobacter* (Bae *et al*., 2005; Weon *et al*., 2006, 2007; Romanenko *et al*., 2008). However, strain 2-5^T contained $C_{16:0}$ ω7*c*/16:1 ω6*c* and $C_{19:0}$ cyclo ω8*c*, which differences with the reference strains. The predominant polar lipid of strain 2-5^T were phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglyceroal (DPG) (Supplementary Fig.S3). The DNA G+C content of strain 2-5^T was 63.8 mol%. This value is within the range reported for the genus *Lysobacter* (Christensen and Cook, 1978; Aslam *et al*., 2009).

3.3 Phylogenetic Analysis and DNA-DNA Hybridization

An almost-complete 16S rRNA gene sequence of strain 2-5^T consisting of 1436 bp was obtained. The phylogenetic tree shows that strain 2-5^T clusters within the genus *Lysobacter* in the class Gammaproteobacteria (Fig.1). 16S rRNA gene sequence analysis revealed that strain 2-5^T was related most closely to *L. concretionis* Ko07^T (97% similarity), *L. arseniciresistens* ZS79^T (96%), and *L. defluii* APB-9^T (96%). Based on the above results, DNA-DNA hybridization experiments were carried out between strain 2-5^T and *Lysobacter concretionis* Ko07^T. The value obtained was 23%, which is significantly below the value of 70% proposed by Wayne *et al*. (1987) for species discrimination.

3.4 Toxonomic Conclusion

It is clear from the 16S rRNA gene sequence comparison and DNA–DNA hybridization data that strain 2-5^T represents a novel species of the genus *Lysobacter* (Wayne *et al*., 1987). In addition, strain 2-5^T was positive for L-Arabinose, D-Mannitol, N-Acetylglucosamine, Maltose, and Trisodium citrate, differs from the type strain *L. concretionis* Ko07^T. Based on the phenotypic, phylogenetic and genomic evidence, strain 2-5^T was identified as a novel species of the genus *Lysobacter*, for which the name *L. hymeniacidonis* sp. nov. is proposed.

3.5 Description of *Lysobacter hymeniacidonis* sp. nov.

Lysobacter hymeniacidonis (hy.me.ni.a.ci´do.nis. N.L. gen. n. *hymeniacidonis* of *Hymeniacidon*, the generic name of the marine sponge *Hymeniacidon sinapium*, the source of the type strain).

Cells are Gram-negative, aerobic, non-spore forming, non-motile (but show gliding activity) and rod-shaped, about 0.2–0.3 μm wide and 1.1–1.2 μm long. Colonies grown on LB agar are convex, circular, smooth, non-transparent and pale yellow after 3 d of incubation at 30℃. Grows at 15–37℃ (optimal 28–30℃); no growth occurs below 4℃ or above 45℃. The pH range for growth is 6.0–8.0 (optimal pH 7.0). Growth occurs in the absence

of NaCl and no growth occurs in 8.0% NaCl. Oxidase and catalase-positive. Positive for acid production from glucose, but negative for reduction of nitrates to nitrites. Hydrolyses aesculin, gelatin and casein, but not starch and chitin. Positive for citric acid utilization and acetoin production (Voges–Proskauer reaction). Activities of beta-

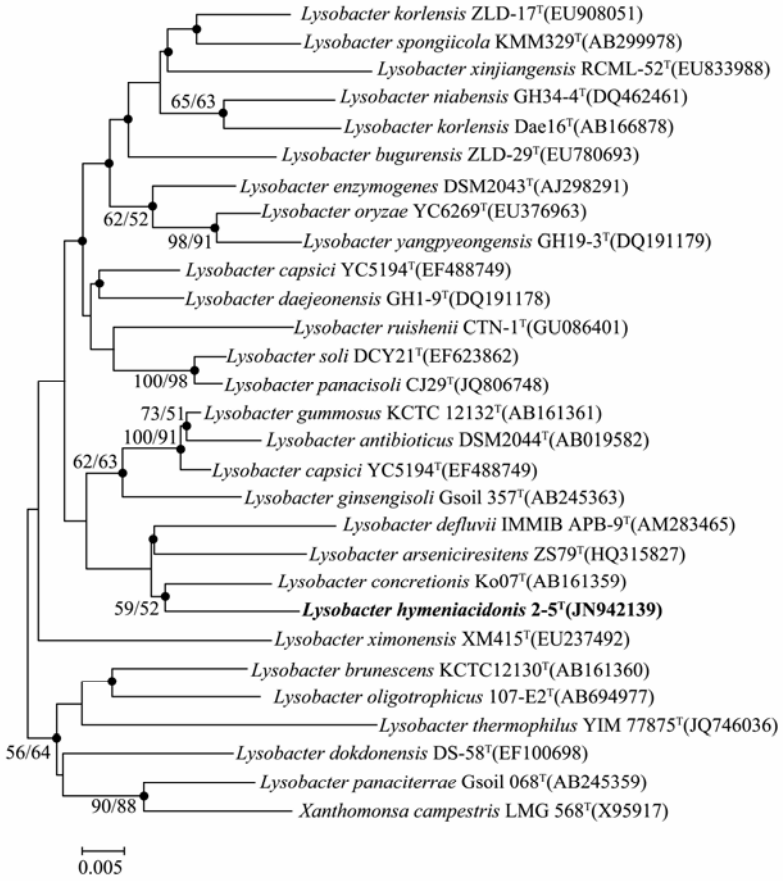

Fig.1 Phylogenetic tree of strain 2-5^T and the type strains of related taxa based on 16S rRNA gene sequences. The tree was reconstructed using the neighbor-joining (NJ) and maximum-likelihood (ML) methods and numbers at nodes represent bootstrap percentages (NJ/ML, based on 1000 resamplings). Filled circles indicate genetic branches that were present in both the NJ and ML trees. GenBank accession numbers are given in parentheses. Bootstrap values over 50% are shown at branching points. Bar, 0.005 substitutions per nucleotide position.

galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease and tryptophan deaminase are negative (API 20 E). Negative for assimilation of dextrin, tween40, tween80, I-erythritol, L-fruc- tose, D-galactose, dextrinose, D-mannose, D-allulose, sucrose, methyl pyruvate, succinic acid methyl, beta- phenylglycollic acid, itaconic acid, alpha-oxo acid, sebacic acid, succinic acid, bromosuccinic acid, D-alanine, L-histidine, D-serine, gamma-amino butyric acid, rocanic acid, thymidine, phenyl ethylamine, putrescine, 2-amino ethanol, 2, 3-butanediol, glycerol, glycerol phosphate, D-glucose-1-phosphate, D-glucose-6-phosphate. Utilizes alpha-Cyclodextrin, glycogen, N-acetylgalactosamine, N-acetylglucosamine, ribitol, L-arabinose, D-arabitol, D-cellobiose, D-fructose, D-glucose, m-inositol, alpha-D-lactose, lactulose, maltose, D-mannitol, D-melibiose, beta-methyl-D-glucoside, D-raffinose, L-rhamnose, D-sorbitol, D-trehalose, turanose, xylitol, acetic acid, cis-aconitic acid, citric acid, formic acid, D-galactose acid lactone, D-galacturonic acid, D-gluconic acid, beta-methyl glucoside, D-glucuronic acid, alpha-hydroxy butyric acid, beta-hydroxy butyric acid, gamma-hydroxy butyric acid, alpha-oxo-butanoic acid, alpha-oxo-pentanedioic acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharinic acid, succinamic acid, glucuronamide, L-alanyl amine, L-alanine, L-alanylglycine, L-asparagine, glycyl aspartic acid, glycyl glutamic acid, hydroxy proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, L-serine, L-threonine, D, L-carnitine, inosine, uridine (API 20 NE and Biolog GN2).

The major cellular fatty acids of strain 2-5^T were iso-$C_{16:0}$, iso-$C_{15:0}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{11:0}$ 3-OH; detailed fatty acid compositions are given in Table 2. The polar lipids consist of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglyceroal. The genomic DNA G+C content of the type strain is 63.8 mol%. The type strain 2-5 ^{T} (=CGMCC 1.12190^T = JCM 18137^T), was isolated from a crude oil-contaminated marine sponge in Dalian, China.

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ent Program of the Chinese Academy of Sciences (No. A1097) and the Ningbo Natural Science Foundation of China (2011A610028).

Supplementary

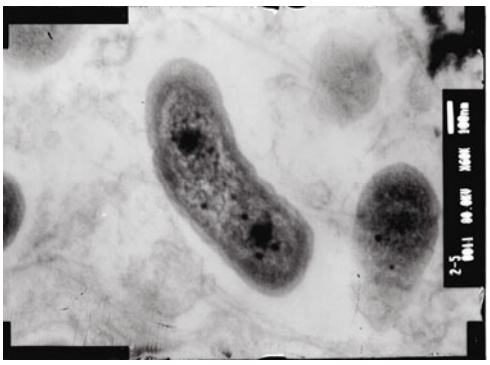

Fig.S1 Transmission electron micrograph of strain 2-5.

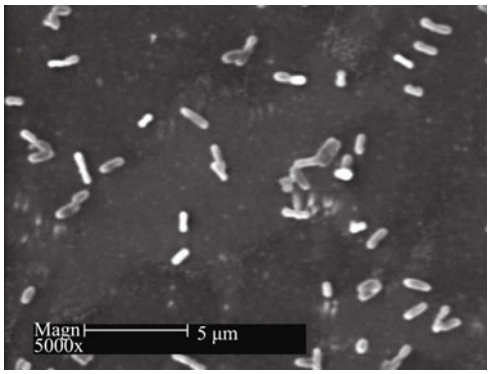

Fig.S2 Scanning electron micrograph of strain 2-5.

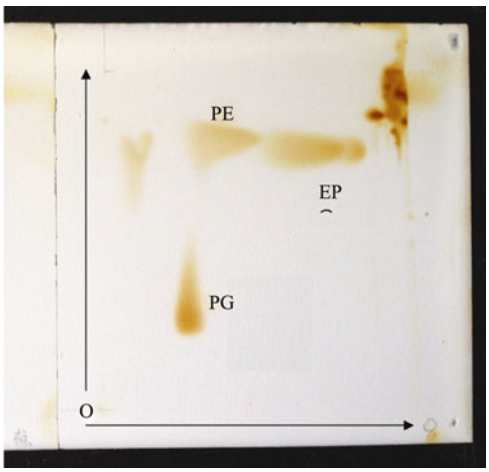

Fig.S3 Two-dimensional TLC of polar lipids of strains 2-5^T stained with 5% ethanolic molybdophosphoric acid. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol.

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