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Bacillus spongiae sp. nov., isolated from sponge of Jeju Island

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A Gram-reaction-positive, strictly aerobic, motile, endospore-forming, and rod-shaped bacterial strain designated 135PIL107-10^T was isolated from a sponge on Jeju Island, and its taxonomic position was investigated using a polyphasic approach. Strain 135PIL107-10^T grew at 20–37°C (optimum temperature, 25°C) and pH 6.0–10.0 (optimum pH, 6.0) on marine and R2A agars. Based on 16S rRNA gene phylogeny analysis, the novel strain formed a new branch within the genus *Bacillus* of the family *Bacillaceae*, and formed clusters with *Bacillus thaohiensis* NHI-38^T (96.8%), *Bacillus fengqiuensis* NPK15^T (96.7%), and *Bacillus songklensis* CAU 1033^T (96.7%). Lower sequence similarities (97.0%) were found with the type strains of all other recognized members of the genus *Bacillus* (95.6–96.8% similarity). The G + C content of the genomic DNA was 43.6 mol%. The predominant respiratory quinone was menaquinone-7 and the major fatty acids were iso-$C_{15:0}$ and iso-$C_{17:1}\omega10c$. The overall polar lipid patterns were diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. The diagnostic diamino acid in the cell-wall peptidoglycan was *meso*-diaminopimelic acid. The isolate therefore represents a novel species, for which the name *Bacillus spongiae* sp. nov. is proposed, with the type strain 135PIL107-10^T (= KACC 19275^T = LMG 30080^T).

Keywords: *Bacillus spongiae*, 16S rRNA gene, polyphasic taxonomy, sponge

Introduction

The genus *Bacillus* was first described by Cohn (1872) and the type species is the *Bacillus subtilis*. Members of the genus *Bacillus* are characterized by Gram-stain-positive, oval to rod-shaped cells, aerobic or facultative anaerobic growth, and endospore formation (Täubel *et al*., 2003; Zhang *et al*., 2010; Kosowski *et al*., 2014). At the time of writing, the genus *Bacillus* includes more than 337 species with validly published names isolated from various environments, including the following recently described species: *Bacillus zeae* (Kämpfer *et al*., 2017) and *Bacillus terrae* (Díez-Méndez *et al*., 2017). Some common taxonomic characteristics of the affiliates of *Bacillus* are Gram-staining-positive, rod-shaped, spore-forming, and containing MK-7 as the major menaquinone and phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), and phosphatidylethanolamine (PE) as the major polar lipids. The major cell wall peptidoglycan is *meso*-diaminopimelic acid. Most strains in this genus have iso-$C_{14:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$, or anteiso-$C_{17:0}$ as major fatty acids. The DNA G + C content ranges from 36 to 52 mol%.

In this study, we evaluated the biodiversity of marine microorganisms and isolated salt-tolerant bacterial strains. We selected marine water and sponge as samples. Many novel bacterial strains were detected in the marine sponge sample, including one novel strain designated as 135PIL107-10^T, which was characterized.

Materials and Methods

Strain isolation

Strain 135PIL107-10^T was isolated from sponge located in Jeju Island (33°11′29.7″N 126°22′42.9″E). The samples were dissolved in sterilized water, serially diluted, and spread onto marine agar medium. The plates were incubated at 30°C for 2 weeks. Single colonies were purified by subculture, and then transferred to new marine agar plates. Strain 135PIL107-10^T was detected and routinely cultured on marine agar medium at 30°C and preserved in marine broth supplemented with 20% glycerol (w/v), at -80°C.

Phenotypic, hydrolysis, and biochemical characteristics of strain 135PIL107-10^T

To phenotypically characterize strain 135PIL107-10^T, standard tests were performed according to the proposed minimal standards for the description of aerobic and endospore-forming bacteria (Logan *et al*., 2009).

The Gram reaction was described by Buck (1982) by using a non-staining method. Cell shape and size were determined by transmission electron microscopy (JEN1010 80 kV, JEOL). Cell motility was analyzed by light microscopy (Optiphot-2, Nikon) at 1000 × as described by Perry in 1973, with cells grown on marine agar for 2 days at 30°C. Cells grown on marine agar for 1 day were used for catalase, oxidase (Cappuccino and Sherman, 2002), biochemical, and phenotypic tests as an inoculum. Biochemical phenotypic tests were conducted using API 20NE, API ID 32GN, and API ZYM kits according to the manufacturer's instructions (bioMérieux). General enzyme activities for the hydrolysis of Tween-80,

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The NCBI GenBank accession number for the 16S rRNA gene sequence of strain 135PIL107-10^T is KY451772.

DNA, starch, casein, and carboxymethyl-cellulose were performed as described by Atlas (1993) and Ten *et al.* (2004). After 7 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 at intervals of 0.5 pH units), agar media (nutrient agar, trypticase soy agar, and MacConkey agar from Difco) were assessed on marine medium at 30°C after 7 days of incubation. Supplemented R2A broth medium containing 1–10% NaCl (w/v at intervals of 1% unit) was used in the salt tolerance test after 7 days of incubation.

Phylogenetic analysis

Using a genomic DNA extraction kit (Macrogen), the DNA of strain 135PIL107-10^T was extracted and subjected to PCR amplification. After PCR amplification of the 16S rRNA gene, the purified PCR product was sequenced, as described by Kim *et al.* (2015). The nearly full-length 16S rRNA gene sequence was assembled using SeqMan software (DNASTAR). 16S rRNA gene sequences of the related taxa were obtained from the GenBank and EzBiocloud [http://www.ezbiocloud.net] server (Yoon *et al.*, 2017). Clustal_X program was used for multiple sequence alignment (Thompson *et al.*, 1997) and gaps were edited in the BioEdit program (Hall, 1999). A Kimura two-parameter model (Kimura, 1983) was used to calculate evolutionary distances. The phylogenetic trees [maximum-parsimony (Fitch, 1971), neighbor-joining (Saitou and Nei, 1987), and maximum likelihood] were constructed with the MEGA 6 Program (Tamura *et al.*, 2013) with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Chemotaxonomic analysis

Quinone, fatty acid, polar lipid, and cell wall peptidoglycan analysis : After growth on marine agar for 2 days at 25°C, the cell biomass of strain 135PIL107-10^T was obtained for quinones. Quinones were first extracted with chloroform/methanol (2:1, v/v) and then evaporated under a vacuum, followed by re-extraction in n-hexane-water (1:1, v/v). The purified crude quinone [using Sep-Pak Vac Cartridges Silica (Waters)] was analyzed by reverse-phase HPLC (Young Lin Instruments) as described by Hiraishi *et al.* (1996). Fatty acid profiles of strain 135PIL107-10^T and its closest reference strains were determined after growth on marine agar for 48 h at 25°C. Cellular fatty acids were extracted according to the protocol of the Sherlock Microbial Identification System (MIDI, Inc.). Fatty acid methyl esters were then analyzed by gas chromatography (model 6890; Hewlett Packard) using the TSBA library (version 6.1), MIDI system (Sasser, 1990). The polar lipid of strain 135PIL107-10^T was determined as previously described by Minnikin *et al.* (1984). Peptidoglycan analysis of strain 135PIL107-10^T was performed as described by Schleifer and Kandler (1972) by thin-layer chromatography (TLC cellulose Merck KGaA 20620 cm).

DNA G + C content analysis

Genomic DNA of strain 135PIL107-10^T was extracted, purified (Moore and Dowhan, 1995), and enzymatically degraded into nucleosides. The G + C content was then determined as described by Mesbah *et al.* (1989) using reverse-phase HPLC.

Results and Discussion

Physiological characteristics

Cells of strain 135PIL107-10^T were Gram-reaction-positive, strictly aerobic, motile, and endospore-forming rods. After 2 days of culture on marine agar plates, colonies were smooth, transparent, circular, light ivory in color, and 3–4 mm in diameter. On marine agar, 135PIL107-10^T grew at 20–37°C, but not at 15°C and 42°C. The isolate grew on nutrient agar and TSA, but not on MacConkey agar. The phenotypic and chemotaxonomic characteristics that differentiate the strain 135PIL107-10^T from other closely related *Bacillus* species are listed in Table 1.

Phylogenetic analysis

The 16S rRNA gene sequence of strain 135PIL107-10^T consists of 1,396 base pairs and has been deposited in the GenBank database under accession number KY451772. The EzBiocloud (http://www.ezbiocloud.net/identify) server sequence similarity indicated that strain 135PIL107-10^T shared less than 96.8% 16S rRNA gene sequence similarity with all taxa with validly published names of the genus *Bacillus*. The highest sequence similarity of the novel isolate was found with *Bacillus thaohiensis* NHI-38^T (96.8%), *Bacillus fengqiuensis* NPK15^T (96.7%), and *Bacillus songklensis* CAU 1033^T (96.7%). Based on phylogenetic tree analysis, strain 135PIL107-10^T clearly formed a separate branch within the genus *Bacillus* and formed a group with *Bacillus thaohiensis* NHI-38^T (KACC 17216^T), *Bacillus fengqiuensis* NPK15^T (KACC 17216^T), *Bacillus songklensis* CAU 1033^T (KCTC 13881^T), *Bacillus abyssalis* SCSIO 15042^T (DSM 25875^T), *Bacillus gaemokensis* (KCTC 13318^T), and *Bacillus luciferensis* (KCTC 3846^T), as shown in Fig. 1. Thus, based on phylogenetic tree analysis, these six strains were selected as the closest reference strains for comparative analysis with strain 135PIL107-10^T.

Quinone, fatty acid, and polar lipid analysis

The predominant quinone of strain 135PIL107-10^T was menaquinone 7 (MK-7), which is similar to all other members of the genus *Bacillus*. The fatty acid of strain 135PIL107-10^T and closest type strains are listed in Table 2. The major cellular fatty acids were iso-$C_{15:0}$ (67.7%) and iso-$C_{17:1}$ ω10*c* (14.6%). Some qualitative and quantitative differences in fatty acid content were observed between strain 135PIL107-10^T and its phylogenetically closest relatives. The main polar lipids were DPG, PG, and PE; minor polar lipids were unidentified phospholipids (PL1, PL2, PL3, and PL4) and unidentified polar lipids (L1, L2, and L3), as shown in Fig. 2.

DNA G + C content

The DNA G + C content of strain 135PIL107-10^T was 43.6 mol%, which is similar to those of described species in the genus *Bacillus* as shown in Table 1.

Taxonomic conclusion

These pilot data indicate that the strain 135PIL107-10^T shares several common features with members of the genus *Bacillus*, e.g. MK-7 as major quinone and DPG, PG, and PE as major

Table 1. Physiological characteristics of strain 135PIL107-10^T and related type strains of the genus *Bacillus*

Strains: 1, *B. spongiae* 135PIL107-10^T; 2, '*B. thaohiensis*' NHI-38; 3, *B. fengqiuensis* NPK15^T; 4, *B. songklensis* CAU 1033^T; 5, *B. abyssalis* SCSIO 15042^T; 6, *B. gaemokensis* BL3-6^T; 7, *B. luciferensis* SSI061^T.

The data presented here are taken from the current study. In API kit system (ZYM, 20NE, and 32GN), all strains were negative for indole production, acid production from glucose, and assimilation of adipate, L-fucose, caprate, and valerate. Positive for assimilation of D-maltose, acetate, and DL-lactate. In API ZYM kit, all the strains were positive for alkaline phosphatase, acid phosphatase and esterase (C4). Negative for lipase (C14), cystine arylamidase, α-galactosidase, β-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, trypsin, α-mannosidase, and α- fucosidase. +, Positive; w, weak positive; –, negative; ND, not determined.

Characteristics	1	2	3	4	5	6	7
Cell size (μm)	0.4–0.6 × 2.5–4.0	ND	1.2–1.9 × 3.5–4.8[b]	0.6–1.5 × 1.8–4.3[c]	1.1–1.6 × 4.0–6.3[d]	1.5–2.0 × 6.0–7.0[e]	0.4–0.8 × 3.0–6.0[f]
Motility	+	+	+	+	-	+	+
Temperature range	20–37	15–60[a]	20–45[b]	10–45[c]	20–60[d]	15–40[e]	15–45[f]
pH range	6.0–10.0	6.5–9.5[a]	7.0–11.0[b]	4.5–10.0[c]	6.0–10.0[d]	5.0–9.0[e]	5.5–8.5[f]
NaCl range (%)	0–8	0–2[a]	0–2[b]	0–4[c]	0–10[d]	0–6[e]	ND
API 20 NE & ID32 GN tests							
Arginine dihydrolase	-	-	-	+	+	+	+
Esculin hydrolysis	+	-	-	-	+	+	-
Gelatin hydrolysis	+	-	-	+	-	+	+
β-Galactosidase (PNPG)	+	-	-	-	-	-	-
Nitrate reduction to nitrite	+	+	+	+	+	-	+
Urease	+	-	-	-	-	+	+
L-Arabinose	+	+	+	-	w	-	-
N-Acetyl-glucosamine	+	-	+	+	+	+	-
Citrate	+	-	+	+	+	-	-
Glycogen	-	-	-	-	-	+	+
L-Histidine	+	+	-	-	-	-	-
4-Hydroxy-benzoate	+	-	+	-	-	-	-
3-Hydroxy-butyrate	-	+	+	+	+	+	-
Inositol	-	+	+	-	+	+	+
Itaconate	-	-	+	-	-	+	+
2-Ketogluconate	+	-	w	-	w	-	-
5-Ketogluconate	-	-	w	-	w	+	+
Malonate	-	-	+	-	-	+	-
D-Mannose	+	-	-	-	-	-	+
D-Mannitol	+	+	+	+	+	-	-
Propionate	+	-	+	+	+	-	-
L-Proline	+	-	+	+	-	-	+
L-Rhamnose	-	-	-	-	-	+	+
Suberate	-	+	-	-	-	+	+
API 20 ZYM test							
Acid phosphatase	+	-	w	+	-	+	+
a-Chymotrypsin	+	-	w	+	w	w	+
Esterase lipase (C8)	-	+	+	+	+	-	+
α-Glucosidase	-	+	+	-	+	+	+
β-Glucuronidase	-	-	-	-	+	-	-
Leucine arylamidase	w	-	w	+	w	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	-	+	w	+	-
Valine arylamidase	-	+	-	w	-	+	-
Isolation source	sponge	forest soil	sandy loam soil	soil	sediment	sediment	geothermal soil
G + C content (mol%)	43.6	40.7[a]	45.5[b]	41.4[c]	43.1[d]	39.4[e]	33.0[f]

[a, b, c, d, e, f] data from '*B. thaohiensis*' NHI-38 (Van Pham and Kim, 2014); *Bacillus fengqiuensis* NPK15^T (Zhao *et al*., 2014); *B. songklensis* CAU 1033^T (Kang *et al*., 2013); *B. abyssalis* SCSIO 15042^T (You *et al*., 2013); '*B. gaemokensis*' BL3-6 (Jung *et al*., 2010); *B. luciferensis* KCTC 3846^T (Logan *et al*., 2002).

polar lipids. However, 16S rRNA gene sequence, phenotypic, and chemotaxonomic characteristics differentiate the novel isolate from recognized species of the genus *Bacillus* (Tables 1 and 2). Therefore, strain 135PIL107-10^T should be assigned to the genus *Bacillus* as the type strain, for which the name *Bacillus spongiae* sp. nov. is proposed.

Description of *Bacillus spongiae* sp. nov.

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

Cells are Gram-reaction-positive, aerobic, endospore-forming, motile, and long rod-shaped (0.7–0.8 μm in diameter and 5.5–6.5 μm in length) after culture on marine agar for

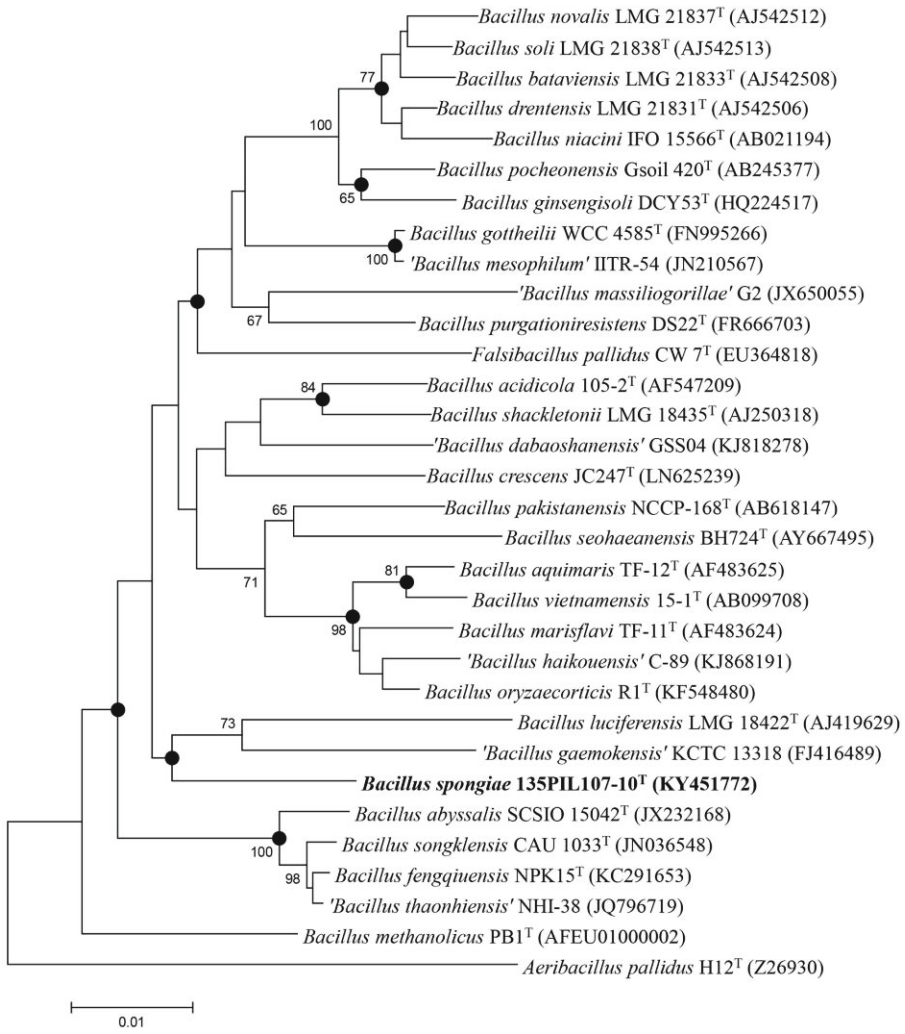

Fig. 1. Phylogenetic relationship between strain 135PIL107-10^T and other related species of the genus *Bacillus* of the family *Bacillaceae*. The tree was constructed using the neighbor-joining method based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1,000 replications) greater than 60% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with maximum-likelihood and maximum-parsimony algorithms. The *Aeribacillus pallidus* H12^T (Z26930) was used as out group. Bar, 0.01 substitutions per nucleotide position.

3 days at 25°C. Colonies grown on the marine agar are smooth, transparent, circular, and light ivory in color and 3–4 mm in diameter after 3 days of incubation on marine agar at 25°C. Growth also occurs on nutrient agar and TSA, but not on MacConkey agar. Cells grow on marine agar at 20–37°C, pH 6.0–10.0, and 0–8% NaCl (w/v). Optimum

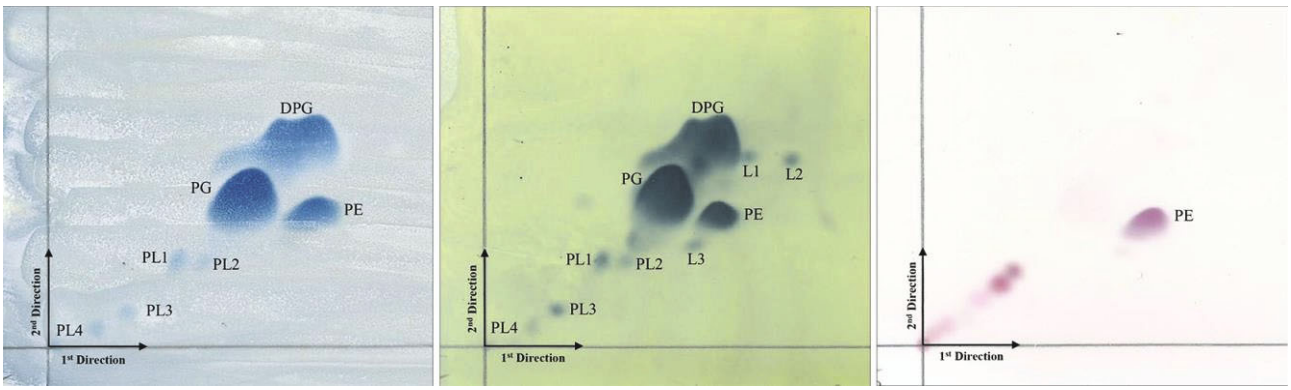

Fig. 2. Two-dimensional TLC of the total polar lipids of 135PIL107-10^T. The TLC plate was stained for total polar lipids with 5% ethanolic molybdophosphoric acid. Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PDE, phosphatidyl-dimethylethanolamine; PME, phosphatidylmono-methyl-ethanolamine; PE, phosphatidyl-ethanolamine; DPG, diphosphatidylglycerol; PL, unidentified phospholipids (PL1, PL2, PL3, PL4); L, unidentified polar lipids (L1, L2, L3).

Table 2. The cellular fatty acid profile of strain 135PIL107-10^T and phylogenetically related species of the genus *Bacillus*
Strain: 1, 135PIL107-10^T; 2, *B. thaohiensis* KACC 17216^T; 3, *B. fengqiuensis* KACC 17216^T; 4, *B. songklensis* KCTC 13881^T; 5, *B. abyssalis* DSM 25875^T; 6, *B. gaemokensis* KCTC 13318^T; 7, *B. luciferensis* KCTC 3846^T. All strains were cultured on marine agar for 48 h at 25°C. Results are presented as percentages of the total fatty acids; fatty acids that represent < 0.5% in all strains are omitted. tr, trace amount.

	1	2	3	4	5	6	7
Saturated							
$C_{12:0}$	1.9	0.6	1.2	1.1	0.9	4.9	2.3
$C_{14:0}$	0.7	2.6	1.6	tr	1.5	3.9	0.8
$C_{16:0}$	1.4	11.1	9.2	1.8	5.6	5.7	tr
$C_{17:0}$	tr	tr	tr	tr	tr	0.8	tr
$C_{18:0}$	tr	tr	0.6	tr	0.5	0.8	9.3
$C_{20:0}$	tr	tr	tr	tr	tr	tr	5.0
Unsaturated							
$C_{16:1}$ *ω7c* alcohol	1.2	1.8	tr	tr	tr	tr	tr
$C_{16:1}$ *ω11c*	1.5	5.9	1.4	tr	0.6	tr	tr
Branched -chain fatty acid							
$C_{11:0}$ iso	tr	tr	tr	tr	tr	0.7	tr
$C_{11:0}$ iso 3OH	tr	tr	tr	tr	tr	0.6	tr
$C_{12:0}$ iso	tr	tr	tr	tr	tr	8.4	tr
$C_{13:0}$ iso	tr	tr	0.5	0.7	0.8	6.0	tr
$C_{14:0}$ iso	-	3.7	3.1	1.0	1.8	12.4	1.0
$C_{15:0}$ iso	67.7	32.9	42.4	53.2	47.9	4.5	7.1
$C_{16:0}$ iso	tr	5.5	3.1	2.0	1.4	16.0	2.3
$C_{17:0}$ iso	7.3	3.3	4.2	6.0	2.9	3.3	tr
$C_{17:0}$ iso 3OH	tr	tr	tr	0.6	tr	tr	tr
$C_{17:1}$ iso *ω5c*	tr	tr	3.1	5.2	4.0	2.1	tr
$C_{17:1}$ iso *ω10c*	14.6	1.5	tr	tr	tr	tr	tr
$C_{18:0}$ iso	tr	tr	tr	tr	tr	1.1	0.8
$C_{18:1}$ iso H	tr	tr	tr	tr	tr	0.8	tr
$C_{19:0}$ iso	tr	tr	tr	tr	tr	tr	3.1
$C_{13:0}$ anteiso	tr	tr	tr	tr	tr	3.1	tr
$C_{15:0}$ anteiso	tr	11.8	13.4	13.3	16.3	3.3	30.4
$C_{17:0}$ anteiso	tr	2.2	1.6	1.8	1.3	2.0	12.3
$C_{17:1}$ anteiso A	tr	tr	1.0	1.4	1.3	1.2	tr
$C_{19:0}$ anteiso	tr	tr	tr	tr	tr	tr	3.0
Hydroxy fatty acids							
$C_{18:0}$ 3OH	tr	tr	0.7	tr	tr	tr	8.8
CYCLO							
1; $C_{14:1}$ *ω5t*/$C_{14:1}$ *ω5c*	tr	tr	tr	tr	0.6	tr	tr
2; $C_{15:1}$ isoH/$C_{13:0}$ 3OH	tr	tr	0.8	0.9	0.8	3.0	tr
3; $C_{14:0}$ 3OH/$C_{16:1}$ iso I	tr	13.7	9.8	7.9	9.7	7.8	tr
4; $C_{16:1}$ *ω7c*/$C_{15:0}$ iso 2OH	0.8	0.6	tr	tr	tr	tr	tr
7; $C_{18:1}$ *ω7c*/*ω9t*/*ω12t*	tr	tr	tr	tr	tr	tr	1.5

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

growth occurs at 25°C and at pH 6.0. Positive for catalase and oxidase. Does not hydrolyze starch, DNA, casein, carboxymethyl-cellulose, cellulose, Tween-80, and skim milk. Carbon assimilation tests as a sole carbon source (API ID 32 GN, API 20 NE) and the enzyme activities (API ZYM) are listed in Table 1. Predominant respiratory quinone is MK-7, and iso-$C_{15:0}$ (67.7%) and iso-$C_{17:1}$ *ω10c* (14.6%) are the major cellular fatty acids (> 10%). The major polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), and phosphatidyl-ethanolamine (PE). The peptidoglycan contained the amino acid *meso*-diaminopimelic acid. The G + C content of genomic DNA is 43.6 mol%.

The type strain, 135PIL107-10^T (= KACC 19275^T = LMG 30080^T) was isolated from a sponge on Jeju Island, Republic of Korea.

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