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Spirosoma pulveris sp. nov., a bacterium isolated from a dust sample collected at Chungnam province, South Korea[§]

Eun Sun Joo[1], Jae-Jin Lee[1], Seho Cha[2], Weonhwa Jheong[3], Taegun Seo[2], Sangyong Lim[4], Sun-wook Jeong[4], and Sathiyaraj Srinivasan[1]*

[1]*Department of Bio and Environmental Technology, College of Natural Science, Seoul Women's University, Seoul 01797, Republic of Korea*
[2]*Department of Life Science, Dongguk University-Seoul, Goyang-si 410-820, Republic of Korea*
[3]*Water Supply and Sewerage Research Division, National Institute of Environmental Research, Incheon 22689, Republic of Korea*
[4]*Radiation Research Division for Biotechnology, Korea Atomic Energy Research Institute, Jeongeup 34057, Republic of Korea*

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Strain JSH 5-14^T, a Gram-negative, non-motile, and curved rod-shaped bacterium, was isolated from a dust sample collected at Nonsan, Chungnam province, South Korea, and was characterized to determine its taxonomic position. Phylogenetic analysis based on the 16S rRNA gene sequence of strain JSH 5-14^T revealed that it belongs to the genus *Spirosoma*, family *Cytophagaceae*, class *Cytophagia*. The highest degree of sequence similarities of strain JSH 5-14^T were found with *Spirosoma liguale* DSM 74^T (97.8%) and *Spirosoma endophyticum* EX 36^T (96.2%). The predominant fatty acids were summed feature 3 (composed of $C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*) and $C_{16:1}$*ω5c*. The major polar lipid was phosphatidylethanolamine, and the predominant respiratory quinone was MK-7. Based on the phylogenetic, chemotaxonomic, and phenotypic data, we propose the strain JSH 5-14^T (=KCTC 42550^T =JCM 30688^T =KEMB 9004-165^T) should be classified as a type strain of a novel species, for which the name *Spirosoma pulveris* sp. nov., is proposed.

Keywords: *Cytophagaceae*, *Cytophagia*, *Spirosoma*, taxonomy

Introduction

The genus *Spirosoma*, belonging to the family *Cytophagaceae*, was originally proposed with *Spirosoma linguale* DSM 74^T as the type species (Larkin and Borrall, 1984). At the time of writing, the genus *Spirosoma* included 8 species, with validly published names (http://www.bacterio.net/spirosoma.html). Members of the genus *Spirosoma* were yellow or orange colored, strictly aerobic or facultatively anaerobic, Gram-stain-negative, and non-motile, with some strains reported to have gliding motility (Baik *et al*., 2007; Ten *et al*., 2009). The chemotaxonomic characteristic properties of the genus *Spirosoma* contains a predominant amount of menaquinone 7 (MK7); the major fatty acids were summed feature 3 (composed of $C_{16:1}$ *ω7c*/ $C_{16:1}$ *ω6c*), $C_{16:1}$*ω5c*, and iso-$C_{15:0}$, and the major polar lipid was phosphatidylethanolamine (Baik *et al*., 2007; Ten *et al*., 2009).

During the course of isolating radiation-resistant bacteria from dust samples, we isolated a strain (designated JSH5-14^T) from a gamma ray-irradiated dust sample collected at Nonsan (GPS; N36°18′71″ E127°9′87″), Chungnam province, South Korea. Strain JSH5-14^T showed Gram-staining-negative and pale-yellow colored colonies on R2A agar (Difco). On the bases of its 16S rRNA gene sequence analysis, strain JSH5-14^T was considered to belong to the genus *Spirosoma*, and the results of a polyphasic taxonomic investigation further confirmed that strain JSH5-14^T represents a novel member of *Spirosoma* species.

Materials and Methods

Isolation of bacterial strain and culture conditions

Strain JSH5-14^T was isolated from a gamma ray-irradiated dust sample collected at Nonsan, Chungnam province, South Korea. A Cobalt-60 gamma irradiator (AECL, IR-79) was used to apply 3kGy gamma radiation, to enrich the radiation resistant bacteria in the dust sample. One gram of the irradiated dust sample was mixed in 10 ml saline [0.85% (w/v) NaCl], diluted serially, and 100 μl of each dilution was spread on a R2A agar (Difco) and incubated at 25°C. Single colonies were transferred onto fresh R2A agar plate and incubated for further 3 days, at 25°C. The 16S rRNA gene of the purified colonies were amplified with colony PCR (Woodman, 2008), and tentatively identified by 16S rRNA gene sequence using the EzTaxon-e (http://eztaxon-e.ezbiocloud.net) (Kim *et al*., 2012).

Strain JSH5-14^T was deposited at the Japan Collection of Microorganisms (JCM 30688^T), Korean Collection for Type Cultures (KCTC 42550^T), and Korea Environmental Microorganisms Bank (KEMB 9004-165^T). The type strain, *Spirosoma linguale*, was obtained from the Korean Agricultural Culture Collection (KACC 12156^T). Both the strains were maintained on R2A agar (Difco), unless otherwise stated.

*For correspondence. E-mail: drsrini@swu.ac.kr; Tel.: +82-2-970-5667; Fax: +82-2-970-5974
The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSH 5-14^T (=KCTC 42550^T =JCM 30688^T =KEMB 9004-165^T) is KP974819.
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

16S rRNA sequencing and phylogenetic analysis

The 16S rRNA gene was amplified using the universal bacterial primer set (Weisburg *et al*., 1991), and subsequently sequenced by Genotech using the 9F, 518F, and 800R universal bacterial primer sets. A nearly complete sequence was compiled with SeqMan software (DNASTAR Inc.) and then compared by use of the EzTaxon-e server. The 16S rRNA sequences of related taxa obtained from GenBank were edited with the BioEdit program (Hall, 1999) and the multiple alignments were performed with the CLUSTAL_X program (Thompson *et al*., 1997). The phylogenetic tree was constructed using the MEGA5 program (Tamura *et al*., 2011). Pairwise distances for the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) were calculated according to the Kimura two-parameter model (Kimura, 1983). A bootstrap analysis with 1,000 replicates was conducted (Felsenstein, 1985). The min-mini heuristic method (Fitch, 1971) with a search factor of one, was applied in a maximum-parsimony (MP) analysis (MEGA 5 Program).

Phenotypic and biochemical characteristics

Gram-staining reaction was performed as described by Gerhardt *et al*. (1994). Cell morphology and motility were examined by light microscopy (Nikon E600) and transmission electron microscopy (Carl Zeiss LEO912AB). Sporulation was examined using Olympus microscope (BX50) after the cells were grown on a modified Schaeffer's medium [KCl 0.1%, $MgCl_2$ 0.01%, $Ca(NO_3)_2$ 1.0 mM, $MnCl_2$ 0.01 mM, $FeSO_4$ 0.001 mM, and Nutrient broth 8 g/L], according to Kempf *et al*. (2005). The pigments were extracted using ethanol (95% v/v) and analyzed using a UV spectrophotometer (UV-2450, Shimazu), with the absorption spectrum between 250 and 800 nm, as described previously (Weeks, 1981; Gosink *et al*., 1998).

Anaerobic growth was evaluated using GasPak jars (BBL) on R2A agar (Difco), nutrient agar (NA, Difco), and trypticase soy agar (TSA, Difco) plates, for 2 weeks at 30°C. Aerobic growth on different media was also assessed on TSA, NA, and TGY (1% tryptone, 0.1% glucose, 0.5% yeast extract) agar at 30°C for 2 weeks. Oxidase activity was evaluated with 1% (w/v) tetramethyl-p-phenylene diamine, and catalase activity was determined by applying 3% (v/v) hydrogen peroxide solution (Smibert and Krieg, 1994). The API 20NE, API 20E, API ID32GN, API 50CH, and API ZYM microtest systems were employed according to the recommendations of the manufacturer (bioMérieux) to study carbon source utilization, enzyme activities, and H_2S production of the strains (JSH5-14^T and *S. linguale* DSM 74^T). Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, and 42°C) was assessed on R2A agar (Difco) for 7 days. Growth at various pH levels (4, 5, 6, 7, 8, 9, and 10) was assessed in R2A broth (MBcell) at 30°C. The pH of the medium was maintained using three buffers (final concentration of 50 mM): acetate buffer (for pH 4.0–5.0); phosphate buffer (for pH 6.0–8.0) and Tris buffer (for pH 9.0–10.0). NaCl tolerance was tested on R2A broth (MBcell) at 30°C that had been supplemented with 0–10% (w/v) NaCl (at 1% intervals).

Gamma and UV radiation resistance analysis

The survival of bacterial strains after exposure to gamma and UCV radiation was measured with positive control (*Deinococcus radiodurans* DSM 20539^T) and negative control (*Escherichia coli* KCTC 1116) cells, cultured in TGY broth (Difco) (Kampfer *et al*., 2008). The cells were irradiated with a Cobalt-60 based gamma irradiator, and the irradiation strength was approximately 100 kCi (3.7 PBq) at a dose rate of 70 Gy/min. For the ultraviolet radiation resistance, a UVC ultraviolet crosslinker (UVP, CX-2000) at 254 nm was used. After the radiation exposure, the cells were serially diluted with saline (0.85% NaCl), spotted on TGY agar plates, and incubated at 30°C for 3 days. The colonies were counted and the survival rate was calculated as described previously (Srinivasan *et al*., 2012a, 2012b; Im *et al*., 2013; Selvam *et al*., 2013).

Chemotaxonomic and genomic analyses

To analyze the cellular fatty acid methyl esters (FAME), the cells were grown on R2A for 3 days at 25°C. Two loops of well-grown cells were harvested, and the fatty acid methyl esters were prepared, separated, and identified (Sherlock version 6.01; data base TSBA6; MIDI, Inc.) (Sasser, 1990). For the analysis of quinone and polar lipids, freeze dried cells were prepared, after culturing on R2A for 3 days at 25°C. Isoprenoid quinones were extracted, purified via thin-layer chromatography (TLC), and analyzed by HPLC, as described previously (Collins and Jones, 1981; Shin *et al*., 1996). Polar lipids were extracted as described by Minnikin *et al*. (1984), and identified using two-dimensional thin-layer chromatography (TLC) (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). The first mobile phase for TLC development was chloroform/methanol/water (65:25:4, v/v/v), and the second mobile phase was chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). The appropriate detection reagents for each type of polar lipids were used as previously described (Lee *et al*., 2014).

DNA-DNA hybridization was performed according to Ezaki *et al*. (1989), with five replications. The highest and lowest values were excluded, and the remaining three values were utilized. To determine the G+C content, genomic DNA was extracted, purified with the Genomic-tip system 100/G (QIAGEN), enzymatically degraded into nucleosides, and analyzed using reverse-phase high performance liquid chromatography (HPLC) (Tamaoka and Komagata, 1984; Mesbah *et al*., 1989).

Results and Discussion

Morphological and phenotypic characteristics

Cells are pale-yellow colored, Gram-staining-negative, non-motile, strictly aerobic, and curved rods, when cultured on R2A agar at 30°C (Fig. 1). Growth was observed at 10–37°C, with optimal growth at 30°C. Strain JSH5-14^T grew at pH values of 7–8. Strain JSH5-14^T showed yellow pigmentation with absorbance peaks at 450.5 nm and 479.5 nm (Supplementary data Fig. S1). Physiological characteristics of strain JSH5-14^T are in the species description, and differential char-

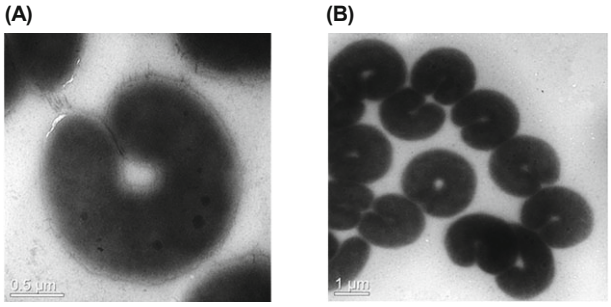

Fig. 1. Transmission electron microscopy of strain JSH5-14^T grown on R2A agar at 25°C for 3 days. Bar, 0.5 μm (A), 1 μm (B).

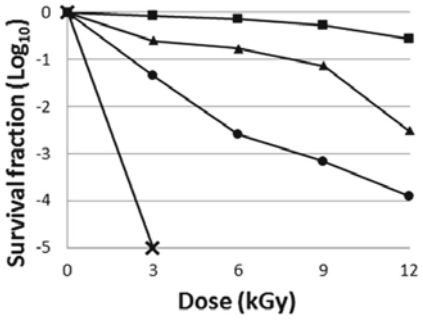

Fig. 2. Representative survival curves for cells of stain JSH5-14^T (*triangle*), *S. linguale* DSM 74^T (*circle*), *D. radiodurans* R1^T (square, positive control), and *E. coli* K12 (cross, negative control) following their exposure to gamma radiation. The y-axis shows survival on a logarithmic scale.

Table 1. Differential characteristics between strain JSH5-14^T and *S. linguale* DSM 74^T

Strains: 1, JSH5-14^T; 2, *S. linguale* DSM 74^T. All data were obtained in this study. Both strains are Gram-negative, non-motile, yellow colored colonies. +, positive; -, negative; w, weakly positive.

Characteristic	1	2
Size (μm)		
Width	3.0–4.0	4.0–5.0
Length	1	1
Growth at pH 8	W	-
Enzyme activity		
N-Acetyl-β-glucosaminidase	+	w
Acid phosphatase	+	-
α-Chymotrypsin	+	w
Cystine arylamidase	W	-
Esterase (C4)	W	-
Esterase (C8)	W	-
β-Galactosidase (ONPG)	+	-
α-Glucosidase	+	-
β-Glucosidase	W	-
Leucine arylamidase	+	w
α-Mannosidase	W	-
Valine arylamidase	+	w
Fermentation		
Amygdalin	+	w
Arbutin	+	w
D-Fructose	W	+
D-Galactose	W	+
Gentiobiose	W	+
Inulin	-	w
Melezitose	W	+
D-Melibiose	W	+
D-Raffinose	W	+
Starch	W	+
D-Trehalose	W	+
D-Xylose	+	w
N-Acetylglucosamine	+	w
Assimilation		
L-Arabinose (20NE)	W	-
L-Arabinose (32GN)	+	w
Gluconate	W	-
N-Acetyl-D-glucosamine	-	w
G+C content (mol%)	49.2	48.6

acteristics between strain JSH5-14^T and *S. linguale* DSM 74^T are shown in Table 1.

Gamma and UV radiation resistance analysis

UV or gamma radiation causes cellular damage, which leads to the cell death in prokaryotic and eukaryotic cells (Ignacio *et al.*, 2013; Kang *et al.*, 2013). Several ionizing radiation resistant bacteria have nucleotide excision repair pathways, which prevent the DNA damage during radiation (Daly, 2009). Strain JSH5-14^T, isolated from gamma-ray irradiated dust sample, showed resistance against gamma and UV radiation. Strain JSH5-14^T has moderate radiation resistance, compared with the control strains *Deinococcus radiodurans* R1^T and *Escherichia coli* K-12^T (Figs. 3 and 4).

Phylogenetic analysis

The 16S rRNA gene sequence of strain JSH5-14^T was a continuous stretch of 1405 nucleotides. Strain JSH5-14^T belongs to the class *Cytophagia*, order *Cytophagales*, and family *Cytophagaceae*. The highest degree of sequence similarity of strain JSH5-14^T was found with the *Spirosoma* species, namely *S. linguale* DSM 74^T (97.8%, Migula, 1894), *S. endophyticum* EX 36^T (96.2%, Fries *et al.*, 2013), *S. radiotolerans* DG5A^T (95.3%, Lee *et al.*, 2014), *S. luteum* DSM 19990^T (94.7%, Finster *et al.*, 2009), and *S. spitsbergense* DSM 19989^T

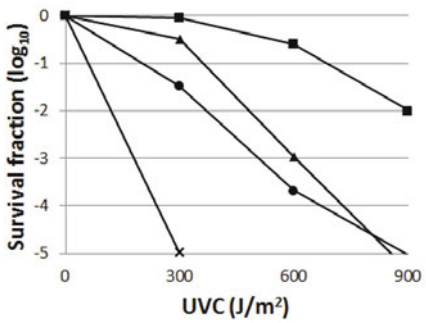

Fig. 3. Representative survival curves for cells of stain JSH5-14^T (*triangle*), *S. linguale* DSM 74^T (*circle*), *D. radiodurans* R1^T (square, positive control), and *E. coli* K12 (cross, negative control) following their exposure to UVC. The y-axis shows survival on a logarithmic scale.

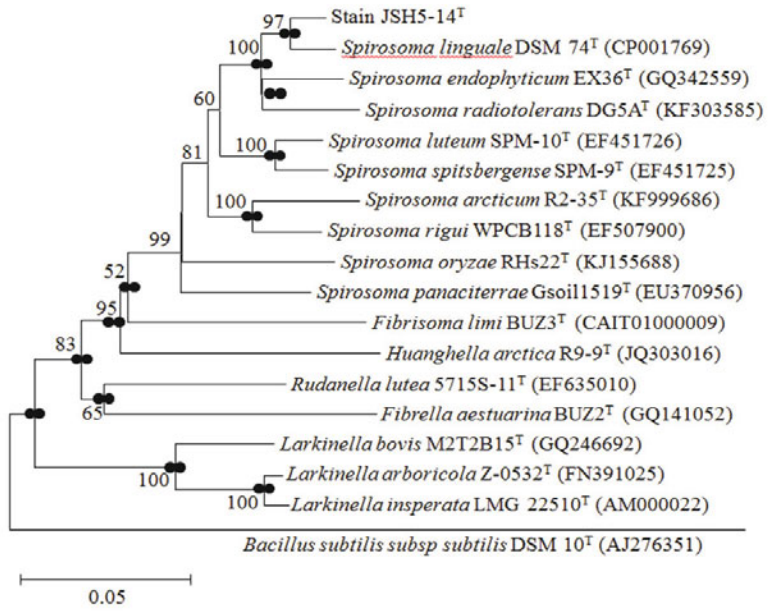

Fig. 4. A neighbor-joining (NJ) phylogenetic tree based on the 16S rRNA gene sequence of strain JSH5-14^T and the representatives of related taxa. Numbers at the nodes indicate the bootstrap values (greater than 50%) expressed as percentage of 1,000 replicates. Single closed circles indicate the corresponding nodes recovered by maximum-parsimony (MP) or maximum-likelihood (ML) algorithm, and double closed circles indicate the corresponding nodes recovered by both maximum-parsimony and maximum-likelihood algorithms. Bar represents 0.05 substitutions per nucleotide position.

(94.5%, Finster *et al.*, 2009). The phylogenetic tree constructed with Neighbor-Joining and Maximum-Parsimony algorithms showed that the strain JSH5-14^T clearly belonged to the genus *Spirosoma* lineage (Fig. 4).

Chemotaxonomic and genomic analyses

The predominant cellular fatty acids of strain JSH5-14^T were summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$) (28.7%), $C_{16:1}\omega5c$ (24.9%), and iso-$C_{15:0}$ (14.4%). Minor fatty acids of strain JSH5-14^T were iso-$C_{17:0}$ 3OH (5.3%), anteiso-$C_{15:0}$ (4.6%), $C_{16:0}$ (4.5%), iso-$C_{15:0}$ 3OH (3.2%), summed feature 9 (iso-$C_{17:1}\omega9c$/10-methyl $C_{16:0}$) (2.5%), iso-$C_{13:0}$ (2.2%), iso-$C_{17:0}$ (1.3%), and summed feature 4 (anteiso B-$C_{17:1}$/iso I) (1.2%). Strain JSH5-14^T could be differentiated from *S. linguale* DSM 74^T based on a larger amount of iso-$C_{15:0}$ (14.5%) fatty acid (Table 2). MK-7 is the predominant quinone of strain JSH5-14^T, similar to the other *Spirosoma* species (Finster *et al.*, 2009; Fries *et al.*, 2013; Lee *et al.*, 2014).

Strain JSH5-14^T contains major amounts of phosphatidylethanolamine (PE), various unknown polar lipids (L_{1-2}), unknown aminolipids (AL), unknown phospholipids (PL), and unknown aminophospholipids (APL_{1-2}) (Supplementary data Fig. S1). Polar lipid profile of strain JSH5-14^T was dominated by PE, which is common in members of the genus *Spirosoma* (Fries *et al.*, 2013; Lee *et al.*, 2014). The G+C content of genomic DNA from strain JSH5-14^T was 49.2 mol%. Strain JSH5-14^T exhibited less than 70% DNA-DNA relatedness with *S. linguale* DSM 74^T (39.2 ± 0.8%; reciprocal hybridization with labeled DNA of strain DSM 74^T is 42.5 ±

Table 2. Cellular fatty acid profiles of strain JSH5-14^T and *S. linguale* DSM 74^T

Strains: 1, JSH5-14^T; 2, *S. linguale* DSM 74^T. Both strains were grown on R2A agar at 25°C for 3 days. tr, trace (less than 1.0%). All values are in %.

Fatty acids	1	2
Saturated		
13:0 iso	2.2	2.3
15:0 iso	14.4	9.5
15:0 anteiso	4.6	3.6
15:0 iso 3OH	3.3	3.8
16:00	4.5	4
16:0 3OH	tr	2
17:0 iso	1.3	tr
17:0 iso 3OH	5.3	6.1
Unsaturated		
16:1 $\omega5c$	24.9	26.5
Summed Feature 3 (16:1 $\omega6c$/16:1 $\omega7c$)	28.7	35.4
Summed Feature 4 (17:1 anteiso B/17:1 iso I)	1.2	1.1
Summed Feature 9 (17:1 iso $\omega9c$/16:0 10-methyl)	2.5	tr

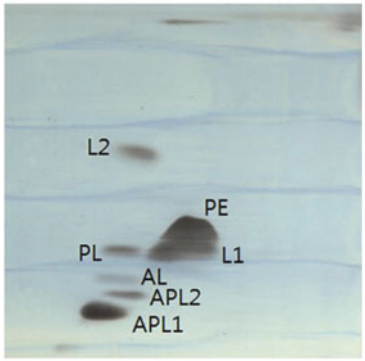

Fig. 5. Two dimensional thin-layer chromatograms, sprayed with molybdophosphoric acid reagent, to identify total polar lipids of strain JSH5-14^T. Ascending solvent system: (I) chloroform/methanol/water (65:25:4, v/v/v); (II) chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). Molybdophosphoric acid (PE, PG, and PL), Ninhydrin (PE), α-Naphthol (GL), and Zinzadze reagent (PE, PG, and PL) were applied to detect the polar lipids. Abbreviations: PE, phosphatidylethanolamine; PL, unknown phospholipids; APL, unknown aminophospholipids; AL, unknown aminolipids; L, unknown polar lipids (The L lipids do not contain a phosphate group, an amino group, or a sugar moiety).

0.7%), and was thus delineated as a different genomic species (Wayne *et al*., 1987; Stackebrandt and Goebel, 1994).

Taxonomic conclusion

Strain JSH5-14^T showed the typical characteristic features of the genus *Spirosoma*, with the presence of predominant respiratory quinone as MK-7; the major fatty acids were summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$), $C_{16:1}\omega5c$, and iso-$C_{15:0}$; the polar lipid profile consisted of major amounts of phosphatidylethanolamine. Strain JSH5-14^T can be distinguished from its closely related species *S. linguale* DSM 74^T, in having the ability to produce acid phosphatase, β-galactosidase and α-glucosidase. Based on the phylogenetic, chemotaxonomic, and phenotypic data, we conclude that strain JSH5-14^T is a representative novel species, for which the name *Spirosoma pulveris* sp. nov., is proposed.

Description of *Spirosoma pulveris* sp. nov.

Spirosoma pulveris (L. n. *pulvis* -eris, dust, powder; L. gen. n. *pulveris*, of dust, referring to the source of the organism) Cells are Gram-negative, strictly aerobic, non-motile, comma-forming, and curved rod-shaped, when grown on R2A agar at 25°C for 3 days. The bacteria could be cultured on TSA (Difco), LB (Difco), NA (Difco), and R2A (Difco). Growth occurred at temperatures of 10–37°C. Strain JSH5-14^T grew well at pH 7–8, and can tolerate up to 3% NaCl (w/v), with optimum growth at 0–1% NaCl (w/v). It showed positive catalase and negative oxidase reactions, and was negative for de-nitrification and indole production. Cells can utilize N-acetyl-D-glucosamine, L-arabinose, D-glucose, D-maltose, D-melibiose, salicin, and D-sucrose as a sole carbon and energy source, but not acetate, L-alanine, caprate, citrate, L-fucose, glycogen, L-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, D,L-3-hydroxybutyrate, myo-inositol, itaconate, 2-ketogluconate (α), 5-ketogluconate, D,L-lactate, malonate, D-mannitol, L-proline, propionate, L-rhamnose, L-serine, D-sorbitol, suberate, and n-valerate. Alkaline phosphatase, esterase (C4), esterase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, Naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase (ONPG), α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, and α-mannosidase are positive. Tests were negative for lipase (C14), β-glucuronidase, and α-fucosidase, but variable results are observed for esterase (C8), naphtol-AS-BI-phosphohydrolase, and valine arylamidase.

The predominant cellular fatty acids of strain JSH5-14^T are summed feature 3 ($C_{16:1}\omega7c/C_{16:1}\omega6c$), $C_{16:1}\omega5c$, and iso-$C_{15:0}$. MK-7 is the predominant quinone. The polar lipid profile consisted of major amounts of phosphatidylethanolamine. The DNA G+C content of the type strain is 49.2 mol%.

The type strain JSH5-14^T (=KCTC 42550^T =JCM 30688^T =KEMB 9004-165^T) was isolated from a dust sample collected from Nonsan (GPS; N36°18′71″ E127°9′87″), Chungnam province, South Korea.

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