Journal of Microbiology (2017) Vol. 55, No. 4, pp. 247–252
DOI 10.1007/s12275-017-6455-y
eISSN 1976-3794
pISSN 1225-8873

Spirosoma luteolum sp. nov. isolated from water[§]

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(Received Sep 8, 2016 / Revised Nov 11, 2016 / Accepted Nov 22, 2016)

A novel Gram-negative and rod-shaped bacterial strain, designated as 16F6E^T, was isolated from a water sample. Cells were yellowish in color and catalase- and oxidase-positive. The strain grew at 10–37°C (optimum at 25°C) but not at 4 and 42°C, and pH 5–7 (optimum at pH 7). It showed moderate resistance to gamma-ray irradiation. Comparative phylogenetic analysis showed that strain 16F6E^T belonged to the family *Cytophagaceae* of the class *Cytophagia*. Furthermore, this isolate showed relatively low 16S rRNA gene sequence similarities (90.7–93.1%) to the members of the genus *Spirosoma*. The major fatty acids were summed feature 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*), $C_{16:1}$ *ω5c*, $C_{16:0}$ N alcohol, and $C_{16:0}$. The polar lipid profile indicated presence of phosphatidylethanolamine, unknown aminophospholipids, an unknown amino lipid, unknown phospholipids, and unknown polar lipids. The predominant isoprenoid quinone was MK-7. The genomic DNA G+C content of strain 16F6E^T was 56.5 mol%. Phenotypic, phylogenetic, and chemotaxonomic properties indicated that isolate 16F6E^T represents a novel species within the genus *Spirosoma*, for which the name *Spirosoma luteolum* sp. nov. is proposed. The type strain is 16F6E^T (=KCTC 52199^T =JCM 31411^T).

Keywords: *Spirosoma*, polyphasic taxonomy, water

Introduction

The genus *Spirosoma* was first reported by Larkin and Borrall in 1978 with *Spirosoma linguale* being the type species of the genus. At the time of writing, the genus *Spirosoma* comprised nine species with validly published names (http://www.bacterio.net/spirosoma.html). Members of the genus *Spirosoma* are Gram-negative and rod-shaped bacteria that contain menaquinone MK-7 as the major quinone, summed feature 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*), $C_{16:1}$ *ω5c*, iso-$C_{15:0}$, and $C_{16:0}$ as the major fatty acids (Finster *et al.*, 2009; Ahn *et al.*, 2014). The novel strain 16F6E^T was isolated from a water sample collected from Han River in Seoul, South Korea. On the basis of the 16S rRNA gene sequence analysis, this isolate was considered to be a *Spirosoma*-like strain. To determine its exact taxonomic position, 16F6E^T was subjected to a detailed investigation using a polyphasic taxonomic approach, including genotypic, chemotaxonomic, and classical phenotypic analyses.

Materials and Methods

Isolation and culture condition

Strain 16F6E^T was isolated from a water sample collected from Han River (37°31′47″ N, 126°55′58″ E) in Seoul, South Korea. The water sample was irradiated with 3-kGy gamma radiation using a cobalt-60 gamma-ray irradiator (MDS Nordion) before the dilution plating method for bacterial isolation. The isolate grew on the R2A agar (Difco) at 25°C for 7 days. The purified colony was routinely cultured on the R2A agar at 25°C and maintained in a 20% glycerol suspension (w/v) at -70°C. The novel strain was tentatively identified by the partial 16S rRNA gene sequence using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net) (Kim *et al.*, 2012). The novel strain 16F6E^T was deposited in the Korean Collection for Type Cultures (KCTC 52199^T) and Japan Collection of Microorganisms (JCM 31411^T). *Spirosoma linguale* KACC 12156^T (Larkin and Borrall, 1984), *S. endophyticum* KACC 17920^T (Fries *et al.*, 2013), *S. panaciterrae* KACC 14098^T (Ten *et al.*, 2009), *S. luteum* KACC 14164^T (Finster *et al.*, 2009), *S. rigui* KACC 13387^T (Baik *et al.*, 2007), *S. spitsbergense* KACC 14163^T (Finster *et al.*, 2009), *S. arcticum* KACC 18577^T (Chang *et al.*, 2014), *S. oryzae* KACC 17324^T (Ahn *et al.*, 2014), and *'S. radiotolerans'* KCTC 32455 (Lee *et al.*, 2014) were used as reference strains. All experiments for polyphasic analyses of the 16F6E^T and reference strains were performed with the same methods and conditions. All strains were aerobically cultivated on the R2A agar unless otherwise mentioned.

Phylogenetic analysis

Total genomic DNA was isolated by the CTAB/NaCl-protocol and the 16S rRNA gene was amplified as described by Frank *et al.* (2008). The purified PCR product was sequenced

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.
The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 16F6E^T is KU758884.

using the bacterial universal primers 27F, 518F, 800R, and 1492R using a DNA sequencing system (8730XL, Applied BioSystems) (Weisburg *et al.*, 1991). The nearly complete 16S rRNA gene sequence (1,440 bp) was assembled with SeqMan software (version 7.1.0; DNASTAR). Phylogenetic neighbors were identified, and pairwise 16S rRNA gene sequence similarity was calculated using the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012). Clustal_X program was used for multiple sequence alignments (Thompson *et al.*, 1997), and phylogenetic trees were constructed using the neighbor-joining (NJ, Saitou and Nei, 1987) and maximum-likelihood (ML) methods by using MEGA5 software (Tamura *et al.*, 2011). The evolutionary distances were calculated using the Kimura three-parameter model in a pairwise deletion manner (Kimura, 1981).

Phenotypic analysis

Gram reaction of strain 16F6E^T was carried out according to Buck (1982). Cell morphology was examined in cells grown for 72 h on R2A plates at 25°C by using a light microscope (BX50, Olympus; magnification: × 1,000) and bio-transmission electron microscope (HT7700, Hitachi). Oxidase activity was indicated by the development of a violet to purple color following addition of 1% (w/v) tetramethyl-*p*-phenylene diamine, and catalase activity was examined by bubble production using 3% (v/v) hydrogen peroxide. Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, and 42°C), pH values (pH 4–10 at 1 pH unit intervals), and salt (NaCl) concentrations (0, 0.5, 1, 2, 3, 4, 5, 10%, w/v) was tested in the R2A medium for 1 week. The effect of pH was determined using acetate (pH 4–6), phosphate (pH 7–8), and Tris (pH 9–10) buffers. Growth on the R2A agar (Difco), nutrient agar (NA, Difco), Luria-Bertani agar (LB, Difco), and tryptic soy agar (TSA, Difco) was also evaluated at 25°C. Biochemical and physiological characteristics were determined using API ZYM, API 20NE, API ID 32 GN, and API 50CH (bioMérieux) according to the manufacturer's instructions. For the analysis of the radiation resistance, survival rate after exposure to gamma radiation was measured and determined as described by Lim *et al.* (2012).

Chemotaxonomic analyses

To analyze the cellular fatty acids, cells of all used strains were harvested after incubation on the R2A agar at 25°C for 72 h. The fatty acid methyl esters were prepared using the classical method of the Sherlock Microbial Identification System (TSBA, version 6.0; MIDI), and analyzed by gas chromatography with the Microbial Identification Software Package (Sasser, 1990). Polar lipids and isoprenoid quinone were extracted according to Minnikin *et al.* (1984). Polar lipids were identified using two-dimensional TLC followed by spraying with the appropriate detection reagents (Komagata and Suzuki, 1987). Isoprenoid quinone was analyzed by HPLC as described previously (Hiraishi *et al.*, 1996). The genomic

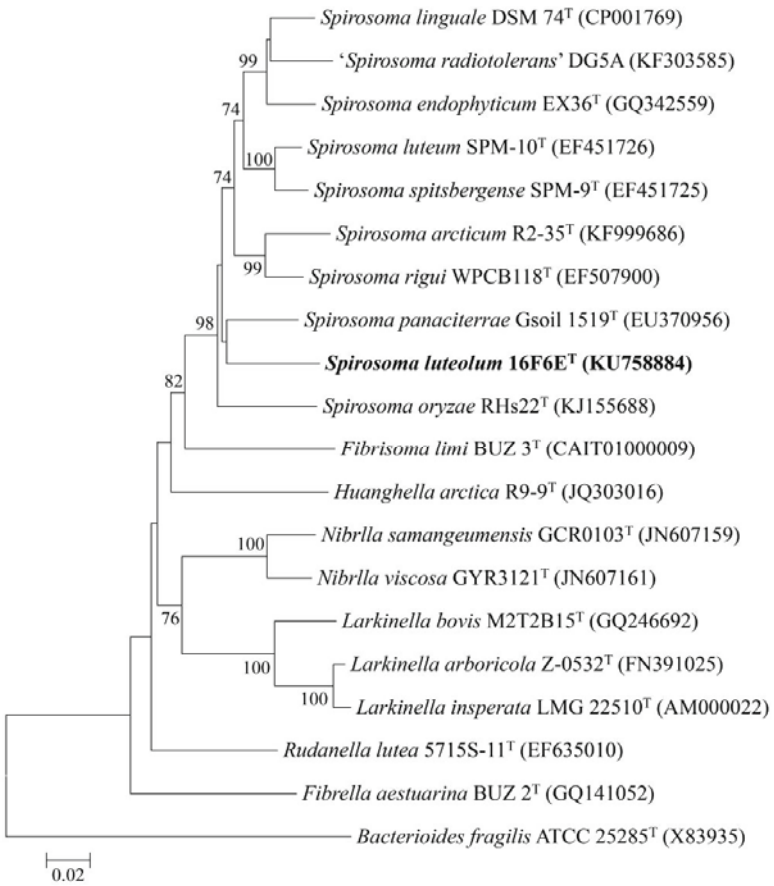

Fig. 1. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic position of strain 16F6E^T among recognized species of the genus *Spirosoma* and other related taxa. Bootstrap values of > 70% (expressed as percentages of 1,000 replications) are shown at branch points. Bar; 0.02 nucleotide substitutions per nucleotide position. *Bacteroides fragilis* ATCC 25285^T was used as outgroup.

DNA G+C content of strain 16F6E^T was analyzed by reverse-phase HPLC (Tamaoka and Komagata, 1984; Mesbah *et al.*, 1989).

Results and Discussion

Phylogenetic analysis

Comparative 16S rRNA gene sequence analyses showed that strain 16F6E^T is phylogenetically affiliated to the members of the genus *Spirosoma*. The similarities of the 16S rRNA gene between strain 16F6E^T and type strains of other *Spirosoma* species ranged from 90.7 to 93.1% (*S. linguale* KACC 12156^T, 93.1%; *S. endophyticum*, 93.0%; S. *panaciterrae*, 92.9%; *S. luteum*, 92.4%; *S. rigui*, 92.3%; *S. spitsbergense*, 91.8%; '*S. radiotolerans*', 91.5%; *S. arcticum*, 91.2%; *S. oryzae*, 90.7%). Such lower than 97% levels of similarity indicated that strain 16F6E^T represented a novel species in the genus *Spirosoma*

Table 1. Differential characteristics of strain 16F6E^T and closely related strains of the genus *Spirosoma*

Strains; 1, 16F6E^T; 2, *S. linguale* KACC 12156^T; 3, *S. endophyticum* KACC 17920^T; 4, *S. panaciterrae* KACC 14098^T; 5, *S. luteum* KACC 14164^T; 6, *S. rigui* KACC 13387^T; 7, *S. spitsbergense* KACC 14163^T; 8, '*S. radiotolerans*' KCTC 32455; 9, *S. arcticum* KACC 18577^T; 10, *S. oryzae* KACC 17324^T.

All strains are rod-shaped bacteria forming yellowish colored colonies. They were grown at 15–25°C, pH 7, NaCl 0–0.5%, but not at 4°C, 42°C, or at pH 4. All strains were catalase positive. They were also positive for acid phosphatase, esterase (C4), β-galactosidase (PNPG), β–glucosidase (esculin hydrolysis), and leucine arylamidase, but not for arginine dihydrolase, β-glucuronidase, lipase (C14), protease (gelatin hydrolysis), or urease. Data on growth at various pH values and NaCl concentrations, DNA G+C contents, and polar lipids of the related *Spirosoma* strains are from Baik *et al.* (2007), Finster *et al.* (2009), Ten *et al.* (2009), Fries *et al.* (2013), Ahn *et al.* (2014), Chang *et al.* (2014), and Lee *et al.* (2014). All other data were obtained in this study.

+, positive reaction; −, negative reaction; w, weakly positive reaction; nd, no data available. Abbreviations: PE, phosphatidylethanolamine; APL, unknown aminophospholipid; AL, unknown aminolipid; PL, unknown phospholipid; GL, unknown glycolipid; DPG, disphosphatidylglycerol; PGL, phosphatidylglycerol; L, unknown lipid.

Characteristic	1	2	3	4	5	6	7	8	9	10
Production of										
Acid from glucose	+	−	−	−	−	−	−	−	−	−
Indole	−	+	−	−	−	−	−	−	−	−
Growth at/on										
Temperature range (°C)	10–37	15–37	15–30	15–37	4–30	15–30	15–30	10–30	15–25	15–30
pH range	5–7	7	5–8	5–9	6–9	6–11	6–9	6–8	7–9	5–10
1% NaCl	+	w	+	+	−	w	−	−	+	w
2% NaCl	w	−	−	−	−	−	−	−	w	−
Tryptic soy agar	+	+	w	+	−	+	−	−	−	+
Luria-Bertani agar	−	−	−	−	−	+	−	−	−	w
Nutrient agar	+	+	+	+	−	+	w	w	−	+
Oxidase activity	+	+	+	+	−	−	w	+	−	−
Enzyme activity:										
N–Acetyl-β-glucosaminidase	+	w	+	+	+	+	+	+	−	+
Alkaline phosphatase	+	+	+	+	+	−	+	+	+	+
α-Chymotrypsin	−	w	+	−	+	−	+	w	−	+
Cystine arylamidase	+	w	+	−	+	−	+	w	−	+
Esterase (C4)	w	w	w	w	w	−	w	w	+	w
Esterase (C8)	+	+	+	+	+	−	+	+	+	+
α-Fucosidase	+	−	−	w	−	−	−	−	−	w
α-Galactosidase	+	+	+	+	+	−	+	w	−	+
β-Galactosidase (ONPG)	+	w	+	w	+	+	+	−	−	+
α-Glucosidase	+	+	+	+	+	+	+	w	−	+
β-Glucosidase	+	w	+	w	+	−	+	−	−	+
α-Mannosidase	+	−	+	w	+	−	+	−	−	w
Naphtol-AS-BI-phosphohydrolase	+	w	+	+	+	−	+	−	+	+
Trypsin	+	+	w	−	+	−	+	w	−	+
Valine arylamidase	+	+	+	+	+	+	+	+	−	+
Assimilation of:										
L-Arabinose	−	−	−	−	−	−	+	−	−	−
D-Glucose	+	+	+	+	w	+	+	−	−	+
D-Maltose	+	+	+	+	+	+	+	−	−	+
D-Mannose	+	+	w	+	+	+	+	−	−	+
L-Malate	w	−	−	−	−	−	−	−	−	−
N-Acetyl-D-glucosamine	+	w	−	w	w	w	+	−	−	+
DNA G+C content (mol%)	56.5	50.2	47.2	50.1	50.2	53.3	49.1	49.1	54.9	57
Polar lipids	PE, APL, AL, PL, L	nd	PE, APL, AL, GL, L	PE, DPG, L	nd	PE, AL, PGL, L	nd	PE, APL, AL	PE, AL, L	PE, APL, AL, PL, L

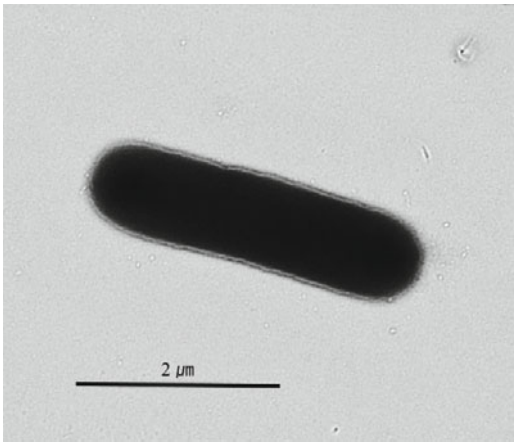

Fig. 2. Transmission electron micrograph of strain 16F6E^T grown on the R2A medium for 3 days at 25°C.

(Wayne *et al.*, 1987; Stackebrandt and Goebel, 1994). In the neighbor-joining (Fig. 1) and maximum-likelihood trees (Supplementary data Fig. S1), the novel strain appeared to be phylogenetically closely related to the members of the genus *Spirosoma*.

Phenotypic characteristics

Strain 16F6E^T was a Gram-negative, rod-shaped (1.0–1.6 μm × 2.8–4.3 μm; Fig. 2), and non-motile bacterium. Cells formed yellowish colored colonies. Growth occurred at 10, 15, 20, 25, 30, and 37°C (optimum at 25°C), pH 5–7 (optimum at pH 7), and 0–2% (w/v) NaCl concentration (optimum at 0% NaCl). Strain 16F6E^T grew well on R2A, NA, and TSA, and weakly on LB. The phenotypic and chemotaxonomic characteristics that differentiated strain 16F6E^T from other *Spirosoma* species are listed in Table 1. The survival rates of strain 16F6E^T following exposure to gamma irradiation at 2 kGy, 4 kGy, 6 kGy, and 8 kGy comprised, respectively, 27.14%, 8.29%, 0.10%, and 0.04%. The survival rate of the *Deinococcus radiodurans* strain R1^T (Brooks and Murray, 1981; White *et al.*, 1999) at 2 kGy was 84.85%, whereas the *Escherichia coli* strain K12 (Kämpfer *et al.*, 2008) did not survive this radiation level (Lim *et al.*, 2006, 2012; Im *et al.*, 2008). Strain 16F6E^T had the moderate survival resistance to gamma irradiation (Supplementary data Fig. S2).

Chemotaxonomic characteristics

The major cellular fatty acids (> 5%) of strain 16F6E^T included summed feature 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*) (33.0%), $C_{16:1}$ *ω5c* (21.9%), $C_{16:0}$ N alcohol (21.4%), and $C_{16:0}$ (9.3%). This fatty acid profile was similar to those of closely related members of the genus *Spirosoma*. However, major quantitative differences between strain 16F6E^T and its closest neighbors were found in the amounts of the abovementioned fatty acids. Apart from the latter differences, strain 16F6E^T could be distinguished from other *Spirosoma* species by a lower amount of $C_{17:0}$ iso 3OH (Table 2). The polar lipid profile of strain 16F6E^T included phosphatidylethanolamine (PE), two unknown aminophospholipids (APL_{1-2}), an unknown amino lipid (AL), three unknown phospholipids (PL_{1-3}), and four unknown lipids (L_{1-6}) (Supplementary data Fig. S3). Phosphatidylethanolamine was detected in strain 16F6E^T and in the majority of type strains of the genus *Spirosoma* (Table 1). Like other members of the genus *Spirosoma* (Finster *et al.*, 2009), strain 16F6E^T contained menaquinone MK-7 as the

Table 2. Cellular fatty acid profiles of strain 16F6E^T and closely related strains of the genus *Spirosoma*

Strains; 1, 16F6E^T; 2, *S. linguale* KACC 12156^T; 3, *S. endophyticum* KACC 17920^T; 4, *S. panaciterrae* KACC 14098^T; 5, *S. luteum* KACC 14164^T; 6, *S. rigui* KACC 13387^T; 7, *S. spitsbergense* KACC 14163^T; 8, '*S. radiotolerans*' KCTC 32455; 9, *S. arcticum* KACC 18577^T; 10, *S. oryzae* KACC 17324^T.

All strains were cultivated on the R2A agar for 72 h at 25°C. Values are percentages of total fatty acids detected. Tr, trace amounts (< 1.0%); –, not detected. *Summed features represent groups of two or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 comprised $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*. Summed feature 4 comprised $C_{17:1}$ iso I and/or $C_{17:1}$ anteiso B. Summed feature 9 comprised $C_{17:1}$ iso *ω9c* and/or $C_{16:0}$ 10-methyl.

Fatty acids	1	2	3	4	5	6	7	8	9	10
Saturated										
$C_{13:0}$ iso	2.2	2.2	1.6	–	–	–	–	2.0	Tr	Tr
$C_{14:0}$	Tr	Tr	Tr	2.0	–	Tr	Tr	1.5	Tr	Tr
$C_{15:0}$ iso	3.3	14.4	3.4	5.9	2.6	3.4	4.9	4.7	7.2	5.6
$C_{15:0}$ anteiso	Tr	4.6	Tr	Tr	1.0	3.9	1.2	1.5	4.2	2.3
$C_{15:0}$ iso 3OH	Tr	3.2	2.4	2.7	Tr	–	3.1	2.1	4.3	2.9
$C_{16:0}$	9.3	4.5	4.0	14.3	7.3	5.8	5.0	16.2	8.6	3.7
$C_{16:0}$ 3OH	Tr	Tr	1.6	3.7	–	–	3.6	1.8	2.5	1.3
$C_{16:0}$ N alcohol	21.4	–	24.0	–	21.8	–	7.7	Tr	12.2	12.1
$C_{17:0}$ iso	–	1.3	–	–	–	–	–	–	1.4	Tr
$C_{17:0}$ iso 3OH	Tr	5.3	4.2	3.1	1.1	3.0	6.8	3.1	11.5	5.4
$C_{18:0}$	1.5	Tr	1.1	9.2	1.3	1.9	Tr	10.4	Tr	Tr
Unsaturated										
$C_{13:1}$ at 12-13	2.1	–	–	–	1.5	1.7	–	–	–	–
$C_{16:1}$ *ω5c*	21.9	24.9	18.1	24.6	16.1	31.8	18.6	19.9	11.5	26.8
Summed Feature 3*	33.0	28.7	37.2	30.6	38.1	48.5	42.4	32.3	26.3	35.8
Summed Feature 4*	Tr	1.2	Tr	–	Tr	–	–	–	1.3	Tr
Summed Feature 9*	–	2.5	–	–	Tr	Tr	–	–	2.0	Tr

major isoprenoid quinone. The genomic DNA G+C content of strain 16F6E^T was 56.5 mol%, which lies within the range observed for the recognized members of the genus *Spirosoma* (Ahn *et al*., 2014) (Table 1).

Taxonomic conclusion

All characteristics determined for strain 16F6E^T are in accordance with those of the genus *Spirosoma.* On the basis of the phylogenetic distance from established *Spirosoma* species, indicated by relatively low 16S rRNA gene sequence similarities (< 93.2%) and a specific combination of phenotypic characteristics, it can be concluded that 16F6E^T is not affiliated to any previously described species of this genus. These results indicated that 16F6E^T should be placed in the genus *Spirosoma* as a representative of a novel species. Therefore, on the basis of the data presented, strain 16F6E^T should be placed in the genus *Spirosoma* as a novel species, for which the name *Spirosoma luteolum* sp. nov. is proposed.

Description of *Spirosoma luteolum* sp. nov.

Spirosoma luteolum (lu.te.o'lum. L. neut. adj. *luteolum*, yellowish, because its colonies exhibit a yellow color).

Cells are Gram-negative and rod-shaped bacterium. Colonies on R2A agar plates are yellowish in color after incubation for 4 days at 25°C. Cells grow at 10–37°C with an optimum at 25°C, but no growth at 4 and 42°C. The pH range for growth is pH 5–7 with an optimum at pH 7. Growth occurs at 0–2% (w/v) NaCl, but no growth occurs at > 2% NaCl in the R2A broth medium. Cells grow well on R2A, NA, TSA, and weakly on the LB agar. Oxidase and catalase activities are positive. Nitrate reduction and indole production are negative (API 20NE). In the API ZYM and API 20NE systems, positive reactions are shown to *N*–acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C8), *α*-fucosidase, *α*-galactosidase, *β*-galactosidase (ONPG), *β*-galactosidase (PNPG), *α*-glucosidase (starch hydrolysis), *β*-glucosidase (esculin hydrolysis), leucine arylamidase, *α*-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase. A weak reaction is observed for esterase (C4). Negative reaction is shown to arginine dihydrolase, *α*-chymotrypsin, *β*-glucuronidase, lipase (C14), protease (gelatin hydrolysis), and urease. In the API 20NE system, growth does not occur with adipate, L-arabinose, arginine dihydrolase, caprate, citrate, gluconate, D-mannitol, and phenyl acetate. In the API 50CH system, acid is produced from *N*-acetyl-glucosamine, D-arabinose, L-arabinose, arbutin, D-cellobiose, esculin, D-fructose, L-fucose, D-galactose, gentiobiose, glucose, α-methyl-D-glucoside, D-lactose, maltose, D-mannose, *α*-methyl-D-mannoside, D-melibiose, D-raffinose, ribose, salicin, starch, D-sucrose, D-trehalose, turanose, D-xylose, and weakly from D-fucose, inulin, D-lyxose, melezitose, D-tagatose, D-xylose, and *β*-methyl-D-xyloside, but not from D-adonitol (ribitol), amygdalin, D-arabitol, L-arabitol, dulcitol (galactitol), erythritol, gluconate, glycerol, glycogen, inositol, 2-ketogluconate, 5-ketogluconate, mannitol, L-rhamnose, sorbitol, L-sorbose, xylitol, or L-xylose. The polar lipid profile contains high amount of phosphatidylethanolamine (PE) and moderate amounts of unknown aminophospholipids (APL_{1-2}), unknown aminolipids (AL), unknown phospholipids (PL_{1-3}), and unknown polar lipids (L_{1-4}). The predominant quinone is MK-7, and the major fatty acids are summed feature 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*), $C_{16:1}$ *ω5c*, $C_{16:0}$ N alcohol, and $C_{16:0}$. The DNA G+C content of the type strain is 56.5 mol%. Cells show a moderate level of resistance to gamma irradiation (8.29% survival rate at 4 kGy). The type strain 16F6E^T (=KCTC 52199^T =JCM 31411^T) was isolated from a water sample collected from Han River in Seoul (37°31′47″ N, 126°55′58″ E), South Korea.

Acknowledgements

This work was partially supported by the Brain Pool Program of 2016 through the Korean Federation of Science and Technology Societies (KOFST) funded by the Ministry of Science, ICT and Future Planning, Republic of Korea (grant 162S-4-3-1727).

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