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Spirosoma montaniterrae sp. nov., an ultraviolet and gamma radiation-resistant bacterium isolated from mountain soil[§]

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A Gram-negative, yellow-pigmented, long-rod shaped bacterial strain designated DY10^T was isolated from a soil sample collected at Mt. Deogyusan, Jeonbuk province, South Korea. Optimum growth observed at 30°C and pH 7. No growth was observed above 1% (w/v) NaCl. Comparative 16S rRNA gene sequence analysis showed that strain DY10^T belonged to the genus *Spirosoma* and was distantly related to *Spirosoma arcticum* R2-35^T (91.0%), *Spirosoma lingual* DSM 74^T (90.8%), *Spirosoma endophyticum* EX36^T (90.7%), *Spirosoma panaciterrae* DSM 21099^T (90.5%), *Spirosoma rigui* WPCB118^T (90.2%), *Spirosoma spitsbergense* DSM 19989^T (89.8%), *Spirosoma luteum* DSM 19990^T (89.6%), *Spirosoma oryzae* RHs22^T (89.6%), and *Spirosoma radiotolerans* DG5A^T (89.1%). Strain DY10^T showed resistance to gamma and ultraviolet radiation. The chemotaxonomic characteristics of strain DY10^T were consistent with those of the genus *Spirosoma*, with the quinone system with MK-7 as the predominant menaquinone, iso-$C_{15:0}$, $C_{16:1}$ *ω5c*, and summed feature3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*), and phosphatidylethanolamine as the major polar lipid. The G+C content of the genomic DNA was 53.0 mol%. Differential phenotypic properties with the closely related type strains clearly distinguished strain DY10^T from previously described members of the genus *Spirosoma* and represents a novel species in this genus, for which the name *Spirosoma montaniterrae* sp. nov. is proposed. The type strain is DY10^T (=KCTC 23999^T =KEMB 9004-162^T =JCM 18492^T).

Keywords: *Cytophagaceae*, *Spirosoma montaniterrae*, radiation-resistant, taxonomy

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The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DY10^T (=KCTC 23999^T =KEMB 9004-162^T =JCM 18492^T) is JQ958375.
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Introduction

The genus *Spirosoma* was first reported with *Spirosoma linguale* as the type species (Larkin and Borrall, 1984) isolated from soil or fresh water. The members of the genus comprise Gram-negative, non-spore-forming, yellow pigmented, and gliding bacteria. At the time of writing, the genus contains eight species (http://www.bacterio.net/spirosoma.html). During the course of investigation on radiation-resistant bacteria in the soil, a Gram-negative, rod-shaped bacterial strain designated DY10^T was isolated. On the basis of 16S rRNA gene sequence similarity analysis, the isolate was assigned to the genus *Spirosoma*. To determine the exact taxonomic position of the strain DY10^T, it was subjected to a polyphasic taxonomic approach including genotypic, chemotaxonomic and phenotypic analyses. The results indicate that strain DY10^T is a novel species candidate and should be placed within the genus *Spirosoma*.

Materials and Methods

Isolation of bacterial strain and culture conditions

Strain DY10^T was isolated from a soil sample (pH 6.6) collected from Mt. Deogyusan (GPS; N 35°51′38″ E 127°44′47″; altitude 1,500 m), Jeonbuk province, Korea. Gamma radiation (5 kGy using a cobal-60 gamma irradiator; point source; AECL, IR-79) was used to irradiate the soil sample prior to isolation of strains. One gram of soil was suspended in 10 ml saline [0.85% (w/v) NaCl] and serially diluted, with 100 µl of each dilution spread on a 1/2 R2A agar and incubated at 30°C. Single colonies on the plate were purified by transferring to fresh R2A agar, and were incubated for 3 days at 30°C. The purified colony was identified by 16S rRNA gene sequence using the EzTaxon-e (http://eztaxon-e.ezbiocloud.net) (Kim *et al.*, 2012).

Phenotypic and biochemical characteristics

Gram reaction was performed according to the classic Gram procedure described by Doetsch (1981). The cell morphology and motility was examined by light microscopy (model BX51; Olympus). The hanging drop motility technique was done after the cells had been grown for 3 days at 30°C on R2A agar (Bernardet *et al.*, 2002). Anaerobic growth was tested by culturing the organisms on R2A, nutrient agar (NA, Difco) and trypticase soy agar (TSA, Difco) plates in GasPak jars (BBL) at 30°C. Oxidase activity was examined by the oxidation of 1% (w/v) tetramethyl-p-phenylene diamine. Catalase activity was determined by measuring bubble production

after applying 3% (v/v) hydrogen peroxide solution. Growth on different media was also assessed on TSA and NA. The pigments of the cells were extracted using 95% ethanol, and a model UV-2450 spectrophotometer (Shimazu) was used to analyze the absorption spectrum between 250 and 800 nm (Weeks, 1981; Gosink *et al.*, 1998).

API 20NE, API 20E, API ID32GN, API 50CH, and API ZYM microtest systems were employed according to the recommendations of the manufacturer (bioMérieux). Growth at different temperatures (4, 10, 15, 20, 25, 30, 37, and 42°C) was assessed on R2A agar (Difco) for 1 week. Growth at various pHs (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 (1% intervals).

Gamma radiation-resistance analysis and ultraviolet survival

The survival rate after exposure to gamma radiation was measured with the early stationary phase (~10^9 CFU/ml) cells in TGY broth (Difco). 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. A positive control (*Deinococcus radiodurans* R1^T; DSM 20539^T) and a negative control (*Escherichia coli* K12; KCTC 1116) were used to compare the resistance of the strain DY10^T (Kampfer *et al.*, 2008). The cells were diluted into micro-well plates and placed on TGY agar plates in duplicate. The colonies were counted and the colony-forming units (CFUs) determined; the survival rate was calculated as described previously (Im *et al.*, 2008; Lim *et al.*, 2012; Srinivasan *et al.*, 2012a, 2012b, 2014; Lee *et al.*, 2014).

Early stationary phase cells (~10^8 CFU/ml) were exposed to different doses of ultraviolet (UV radiation using a model CX-2000 UVC ultraviolet crosslinker (UVP) at 254 nm. After 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 aforementioned positive and negative controls were used to compare the resistance of strain DY10^T. Colonies were counted and survival rate was calculated as described previously (Im *et al.*, 2013; Selvam *et al.*, 2013).

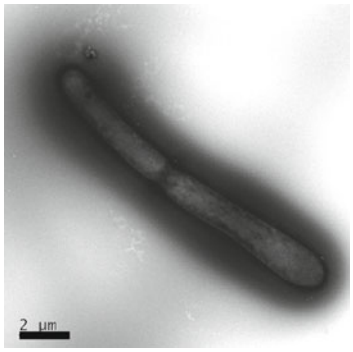

Fig. 1. Transmission electron microcopy of strain DY10^T grown on R2A agar for 3 days at 30°C (Bar, 2 μm)

Phylogenetic analysis

Genomic DNA was extracted using a commercial genomic DNA extraction kit (Solgent) and the 16S rRNA gene was amplified by PCR with 9F/1492R primers (Weisburg *et al.*, 1991). Purified PCR product was sequenced using the 9F, 518F, 785F, and 800R universal bacterial primer set by Genotech. The nearly complete sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR). The 16S rRNA gene sequences of the related taxa were obtained from GenBank and edited using the BioEdit program (Hall, 1999). Multiple alignments were performed with the CLUSTAL_X program (Thompson *et al.*, 1997). The evolutionary distances were calculated using the two-parameter model (Kimura, 1983). Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood methods in MEGA5

Table 1. Comparisons of phenotypic characters of strain DY10^T and closely-related type strains. Taxa: 1, strain of DY10^T; 2, *S. arcticum* LMG28141^T; 3, *S. lingual* DSM 74^T.
All the data obtained in the study and experiments were conducted on same condition. All strains are Gram-negative, aerobic bacterium. +, positive reaction; w, weak positive; -, negative reaction.

Characteristic	1	2	3
Oxidase	+	-	+
Cell shape	rod	rod	helice
Colony color	yellow	orange	yellow
Production of acid from glucose	+	-	+
Growth at			
4°C	-	+	-
30°C	+	-	+
pH 6	+	-	w
pH 9	-	+	-
Enzyme activity			
N-Acetyl-β-glucosaminidase	+	-	-
α-Chymotrypsin	+	-	w
Esterase (C4)	w	+	-
Esterase (C8)	+	+	-
α-Galactosidase	w	-	-
β-Galactosidase (PNPG)	+	-	+
Leucine arylamidase	w	+	-
Protease (gelatin hydrolysis)	-	+	-
Trypsin	+	-	-
Valine arylamidase	+	-	-
Fermentation			
D-Arabinose	+	-	+
D-Fructose	+	-	+
D-Galactose	+	-	+
D-Lactose	+	-	+
Maltose	+	-	+
D-Mannose	+	-	+
D-Raffinose	+	-	+
D-Sucrose	+	-	+
Assimilation			
L-Arabinose	-	-	+
D-Glucose	-	-	+
D-Maltose	-	-	+
DNA G+C content (mol%)	53.0	54.9	49.6

program (Tamura, 2011) with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Chemotaxonomic and genomic analyses

Strain DY10^T was grown on R2A for 2 days at 30°C to perform the fatty acid methyl ester analysis with two loops of third quatrain cells. The fatty acids were methylated, separated, and identified with the Sherlock Microbial Identification System version 6.01 (data base TSBA6; MIDI) as previously described (Sasser, 1990). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), and subsequently analyzed by high-performance liquid chromatography (HPLC) (Collins and Jones, 1981; Shin *et al*., 1996). Polar lipids were extracted according to the procedures described by Minnikin *et al*. (1984) and identified using two-dimensional thin-layer chromatography (TLC) followed by spraying with the appropriate detection reagents (Minnikin *et al*., 1984; Komagata and Suzuki, 1987). To determination genomic DNA G+C content, the purified DNA was enzymatically degraded into nucleosides and analyzed using reverse-phase HPLC, as previously described (Tamaoka and Komagata, 1984; Mesbah *et al*., 1989).

Results and Discussion

Morphological and phenotypic characteristics

Strain DY10^T was yellow colored, Gram-negative, rod-shaped, aerobic, and displayed gliding motility when grown on R2A agar at 30°C for 3 days (Fig. 1). Absorbance peaks were evident at 454.0 nm and 478.0 nm (Supplementary data Fig. S1). Growth occurred between 10–30°C and pH of 6–8. No growth was observed above 1% NaCl (w/v) in R2A. The physiological characteristics of the strain DY10^T was summarized in the species description. The differential characteristics between the strain DY10^T and the closely related strains are shown in Table 1.

Phylogenetic analysis

The 16S rRNA gene sequence of strain DY10^T was a continuous stretch of 1452 nucleotides. On the basis of 16S rRNA gene sequence, strain DY10^T could be assigned to the genus *Spirosoma* and the closest relatives were *Spirosoma arcticum* R2-35^T (91.0%) *S. lingual* DSM 74^T (90.8%), *S. endophyticum* EX36^T (90.7%), *S. panaciterrae* DSM 21099^T (90.5%), *S. rigui* WPCB118^T (90.2%), *S. spitsbergense* DSM 19989^T (89.8%), *S. luteum* DSM 19990^T (89.6%), *S. oryzae* RHs22^T (89.6%), and *S. radiotolerans* DG5A^T (89.1%). The 16S rRNA gene sequence similarity with other members of the *Spirosoma* was ≤ 90.5%. The neighbor-joining phylogenetic tree clearly showed that strain DY10^T clustered with other *Spirosoma* species, which was confirmed in the maximum-parsimony and maximum-likelihood algorithms (Fig. 2).

Chemotaxonomic characteristics

The fatty acid profiles of strain DY10^T consisted of major amounts (>10%) of iso-$C_{15:0}$, $C_{16:1}$ *ω5c*, and summed feature 3 ($C_{16:1}$ *ω7c* / $C_{16:1}$ *ω6c*); and moderate amounts (>5%) of $C_{16:0}$; and minor amounts (<5%) of $C_{14:0}$, anteiso-$C_{15:0}$, $C_{15:0}$ 3OH, iso-$C_{15:0}$ 3OH, $C_{16:0}$ 3OH, iso-$C_{17:0}$ 3OH, at 12-13 $C_{13:1}$, iso G-$C_{15:1}$, $C_{17:1}$ *ω6c*, and summed feature 8 ($C_{18:1}$ *ω7c* / $C_{18:1}$ *ω6c*). The major cellular fatty acids [$C_{16:1}$ *ω5c* and summed feature 3 ($C_{16:1}$ *ω7c* / $C_{16:1}$ *ω6c*)] were common to most *Spirosoma* species, but there were some quantitative differences

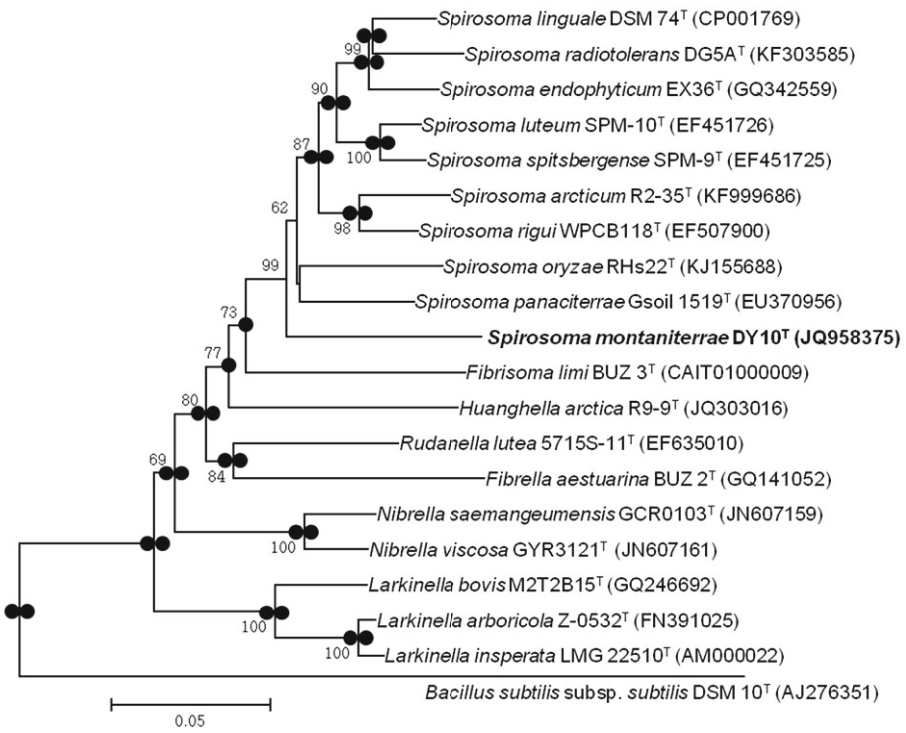

Fig. 2. Phylogenetic tree of strain DY10^T and related *Spirosoma* type strains based on 16S rRNA gene sequence comparisons. Distances were calculated and clustering with the neighbor-joining method was conducted by using the software MEGA5. Bootstrap values of >50% (percentages of 1,000 replications) are shown at branching points. The sequences used for the comparative study are included in parentheses. *Closed circles* indicate the corresponding nodes recovered by maximum-parsimony or maximum-likelihood algorithm.

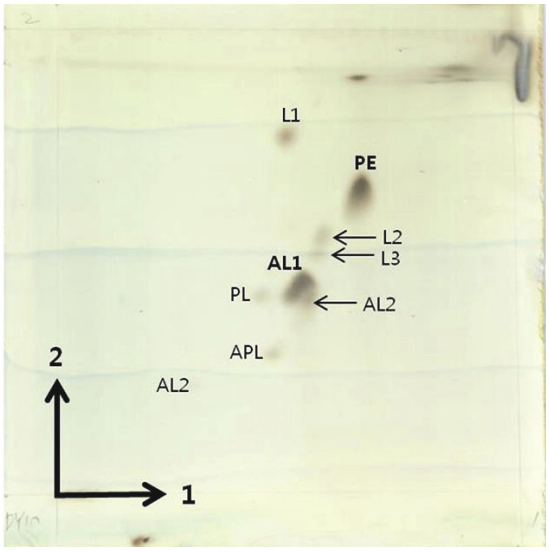

Fig. 3. Two dimensional thin-layer chromatography of total polar lipids of strain DY10^T. Total polar lipids were detected by spraying with molybdophospholic acid. Abbreviations: PE, phosphatidylethanolamine; AL, unknown aminolipids; APL, unknown aminophospholipids; PL, unknown phospholipids; L, unknown polar lipids (L lipids do not contain a phosphate group, an amino group or a sugar moiety)

between the closely related type strains (Chang *et al*., 2014). Strain DY10^T contained MK-7 as the major menaquinone, similar to other *Spirosoma* species (Finster *et al*., 2009; Lee *et al*., 2014). The polar lipid profile of strain DY10^T contained major amounts of phosphatidylethanolamine (PE) and unknown amino lipid (AL), and minor amounts of unknown lipids. Known amino phospholipids were also present (Fig. 3). The DNA G+C mol% of the strain DY10^T was 53.0 mol%, which was within the range of reported *Spirosoma* species (Ten *et al.,* 2009; Fries *et al*., 2013).

Gamma and UV radiation-resistance analysis

The radiation-resistant bacteria were isolated from soil by the redundancy method using irradiated soil samples (Rainey *et al*., 2005). Ionizing radiation produces reactive oxygen species that target the cellular integrity and function of macromolecules both in prokaryotic and eukaryotic cells. Nucleotide excision repair pathway has been reported (Daly, 2009; Ignacio *et al*., 2013; Kang *et al*., 2013), which prevents DNA damage from ionizing radiation. Strain DY10^T was isolated from the gamma-ray irradiated soil sample; it displayed resistance to gamma and UV radiation. Strain DY10^T showed similar UVC and gamma ray radiation curves with the positive strain R1^T (Supplementary data Table S1 and Fig. S2). The highest resistance (70% survival) was obtained to 9kGy gamma radiation, similar that to *D. radiodurans* R1^T. Similarly, strain DY10^T showed highest resistance to UVC radiation (Supplementary data Fig. S2). As expected, the *E. coli* negative control did not survive even the lowest dose of 400 Jm^{-2}.

Taxonomic conclusion

The novel strain DY10^T has the chemotaxonomic characteristics of the genus *Spirosoma* as described Larkin and Borrall (1984), Baik *et al.* (2007), Ahn *et al.* (2014) by the by the presence of MK-7 as the predominant respiratory quinone, phosphatidylethanolamine (PE) as the major polar lipid, $C_{16:1}$ *ω7c*, $C_{16:1}$ *ω5c*, and iso-$C_{15:0}$ as the abundant fatty acids. The phenotypic and biochemical characteristics, which differentiate strain DY10^T from the closely related species (Table 1) clearly distinguishes the strains as a novel species within the genus *Spirosoma*, for which the name *Spirosoma montaniterrae* sp. nov. is proposed.

Description of *Spirosoma montaniterrae* sp. nov., *Spirosoma montaniterrae*

(*montaniterrae*; L. adj. montanus, of a mountain; L. n. terra, soil; N.L. gen. n. *montaniterrae*, of mountain soil, where the type strain was isolated).

Cells are 5 to 15 μm long × 1 to 2 μm wide, Gram-negative, strictly aerobic, motile by gliding, rod-shaped, and produce yellow-colored colonies when grown on R2A at 30°C for 3 days. Cells can grow on NA and R2A (Difco). Growth can occur at 10–30°C, with optimum growth at 30°C. Growth is observed at pH values of 6–8, with optimum growth at pH 7. No growth occurs above 1% NaCl (w/v) in R2A, with optimum growth at 0% NaCl (w/v). Catalase and oxidase are positive, and indole production is negative. Nitrate is not reduced to nitrite, but to di-nitrogen (API 20NE). Cells have a high resistance to UVC and a gamma-radiation, similar to *D. radiodurans* R1^T.

API 50CH system testing revealed that acid is produced with N-acetyl-glucosamine, amidon, amygdalin, D-arabinose, L-arabinose, arbutin, D-cellobiose, esculin ferric citrate, D-fructose, D-fucose, L-fucose, D-galactose, gentiobiose, D-glucose, glycogen, 5-ketogluconate, D-lactose, D-lyxose, D-maltose, D-mannose, D-melezitose, D-melibiose, *α*-methyl-D-glucoside, *α*-methyl-D-mannoside, *β*-methyl-D-xyloside, D-raffinose, L-rhamnose, D-ribose, salicin, L-sorbose, D-sucrose, D-tagatose, D-trehalose, D-turanose, D-xylose, and, L-xylose. Acid is not produced with D-adonitol, D-arabitol, L-arabitol, dulcitol, erythritol, gluconate, glycerol, inositol, inulin, 2-ketogluconate, D-mannitol, D-sorbitol, and xylitol.

In tests with the API ZYM system, enzyme production is positive for *N*-acetyl-*β*-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase (C8), α-galactosidase, *β*-galactosidase (ONPG), α-glucosidase (starch hydrolysis), leucine arylamidase, naphtol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase. Enzyme production is negative for α-fucosidase, *β*-glucosidase, *β*-glucuronidase, lipase (C14), and α-mannosidase.

In tests with the API 32GN and API 20NE system, growth is observed with glycogen. Growth is not observed with acetate, N-acetyl-D-glucosamine, adipate, L-alanine, L-arabinose, caprate, citrate, L-fucose, gluconate, D-glucose, L-histidine, 3-hydroxybenzoate, 4-hydroxybenzoate, D,L-3-hydroxybutyrate, itaconate, 2-ketogluconate, 5-ketogluconate, D,L-lactate, L-malate, malonate, D-maltose, D-mannitol, D-mannose, D-melibiose, myo-inositol, L-rhamnose, D-ribose, salicin, L-serine, D-sorbitol, suberate, D-sucrose, phenyl acetate, L-proline, propionate or n-valerate.

The predominant cellular fatty acids are iso-$C_{15:0}$, $C_{16:1}$ *ω5c*,

and summed feature 3 ($C_{16:1}$ $\omega7c$ / $C_{16:1}$ $\omega6c$). Menaquinone MK-7 is the predominant quinone, and an unknown PE and AL are the major polar lipids. DNA G+C content of the type strain is 53.0 mol%.

The type strain DY10^T (=KCTC 23999^T =KEMB 9004-162 =JCM 18492^T) was isolated from a soil sample collected from Mt. Deogyusan (N 35°51′38″ E 127°44′47″; altitude 1,500 m), Jeonbuk Province, South Korea.

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