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Spirosoma harenae sp. nov., a Bacterium Isolated from a Sandy Beach

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Abstract A Gram-stain-negative, non-motile, non-spore-forming, rod-shaped, aerobic bacterium, designated 15J8-9^T, was isolated from a sandy beach in Jeju Island, South Korea. The isolate was able to grow between 10 and 30 °C, pH 5–8, and in presence of 0–1% (w/v) NaCl. Based on 16S rRNA gene phylogenetic analysis, the novel strain was closely related to members of the genus *Spirosoma* (96.1–90.9% similarities) and showed highest sequence similarity to *Spirosoma panaciterrae* DSM 21099^T (96.1%). The G + C content of the genomic DNA of strain 15J8-9^T was 45.1 mol%. The isolate contained menaquinone MK-7 as the predominant respiratory quinone, phosphatidylethanolamine as the major polar lipid, and summed feature 3 ($C_{16:1}$ *ω6c*/$C_{16:1}$ *ω7c*; 28.0%), $C_{16:1}$ *ω5c* (23.4%), iso-$C_{15:0}$ (13.5%), and $C_{16:0}$ (11.5%) as the major fatty acids that supported the affiliation of strain 15J8-9^T to the genus *Spirosoma*. The isolate could be differentiated clearly from recognized *Spirosoma* species on the basis of several phenotypic, genotypic and chemotaxonomic features. Therefore, strain 15J8-9^T is considered to represent a novel species of the genus the genus *Spirosoma*, for which the name *Spirosoma harenae* sp. nov. is proposed. The type strain is 15J8-9^T (= KCTC 52030^T = JCM 31993^T).

Keywords *Spirosoma* · *Bacteroidetes* · Polyphasic taxonomy

Introduction

The genus *Spirosoma*, belonging to the family *Cytophagaceae*, order *Cytophagales*, class *Cytophagia* and phylum *Bacteroidetes*, was originally proposed with *Spirosoma linguale* DSM 74^T as the type species [21] and the genus description was later emended by Finster et al. [6] and Ahn et al. [1]. At the time of writing, the genus *Spirosoma* comprised fifteen species with validly published names, thirteen of which were directly listed in LPSN (http://www.bacterio.net/spirosoma.html), with the remaining two are awaiting notification [13, 24]. Moreover, several other strains, such as '*Spirosoma fluminis*' 15J17T [22], '*Spirosoma lacussanchae*' CPCC 100624 [25], and '*Spirosoma luteolum*' 16F6E [23] have recently been proposed as novel *Spirosoma* species and are awaiting validation. The type strains of *Spirosoma* species have been recovered from a wide range of natural sources, including air [15, 16], dust [12], fresh water [2, 10, 24], soil [1, 13, 32, 37], plant xylem sap [8], and extreme environments such as high Arctic glacial till [4] or Arctic permafrost soil [6]. Members of the genus *Spirosoma* are Gram-negative, yellow- or orange-pigmented, non-spore-forming, mostly rod-shaped bacteria that are characterized chemotaxonomically as having phosphatidylethanolamine (PE) as the major polar lipid, menaquinone MK-7 as the predominant quinone, and summed feature 3 ($C_{16:1}$ *ω6c*/$C_{16:1}$ *ω7c*), $C_{16:1}$ *ω5c*, $C_{15:0}$ iso, and $C_{16:0}$ as the major fatty acids.

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In the course of screening for novel bacteria, strain 15J8-9^T was isolated from a beach sand sample collected in Jeju Island, Korea. On the basis of a 16S rRNA gene sequence analysis, this isolate was considered to be a *Spirosoma*-like strain. Strain 15J8-9^T was subjected to detailed investigation using a polyphasic taxonomic approach that included genotypic, chemotaxonomic, and phenotypic analyses. Based on the results obtained in this study, we propose that strain 15J8-9^T should be placed in the genus *Spirosoma* as the type strain of a novel species.

Materials and Methods

Isolation of Bacterial Strain and Culture Conditions

Strain 15J8-9^T was isolated from a beach sand sample collected in Jeju Island (29°51′61″ N, 126°27′08″ E), South Korea. The sample was suspended and serially diluted in distilled water. One hundred microliter of each dilution was spread onto R2A agar plates (Difco, USA) and incubated at 25 °C for 1 week. Single colonies on the plates were purified by transfer onto fresh plates, followed by incubation under the same conditions. One yellow colony, designated 15J8-9^T, was routinely cultured on R2A agar at 25 °C and was maintained as a glycerol suspension (20%, w/v) at − 70 °C. The isolate was deposited in the Korean Collection for Type Cultures (KCTC) and the Japan Collection of Microorganisms (JCM). The reference type species *Spirosoma linguale* KACC 12156^T and *Spirosoma panaciterrae* KCTC 22263^T were obtained from the Korean Agricultural Culture Collection and the Korean Collection for Type Cultures, respectively.

Phenotypic and Biochemical Characteristics

Gram reaction of strain 15J8-9^T was examined using a staining method [30]. The morphology of the cells of strain 15J8-9^T, grown for 3 days at 25 °C on R2A agar, was observed under an Olympus light microscope (1000 × magnification) and a Hitachi HT7700 transmission electron microscope. Motility was investigated on 0.5% (w/v) semi-solid R2A agar and gliding motility was assessed by the microscopic hanging drop technique. Catalase activity was determined by assessing the production of bubbles in 3% (v/v) H_2O_2, and oxidase activity was tested using 1% (w/v) tetramethyl-*p*-phenylenediamine [3]. Growth was assessed on R2A agar, Luria–Bertani agar (LB), nutrient agar (NA), and trypticase soy agar (TSA), purchased from Difco, USA. The effect of pH on growth was evaluated in R2A broth using three different buffers (final concentration, 100 mM): sodium acetate buffer (for pH 4.0–6.0), potassium phosphate buffer (for pH 7.0–8.0), and Tris buffer (for pH 9.0–10.0). Growth at 4, 10, 15, 20, 25, 30, 37, and 42 °C was assessed on R2A agar after 7 days of incubation. Salt tolerance was tested in R2A broth supplemented with 0.5, 1, 2, 3, 4, 5, and 10% (w/v) NaCl after 7 days of incubation. Enzyme activities, assimilation of carbon sources, acid production from substrates, and other physiological characteristics were determined by inoculating API ZYM, API 20 NE, API ID 32 GN, and API 50 CH strips according to the manufacturer's instructions (bioMérieux).

16S rRNA Gene Sequencing and Phylogenetic Analysis

For the phylogenetic analysis, the 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primers, 27F and 1492R, as described previously [35], and the purified PCR products were sequenced by Genotech (Daejeon, South Korea). Phylogenetic neighbors were identified, and pairwise 16S rRNA gene sequence similarities were calculated using both the EzTaxon-e server [14] and BLAST search program at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA gene sequences of related taxa were obtained from GenBank and aligned with that of strain 15J8-9^T using the program Clustal X [33]. Gaps and the 5′ and 3′ ends of the alignment were edited manually in BioEdit [9]. Tree topologies were inferred by neighbor-joining (NJ) [28], maximum-likelihood (ML) [5], and maximum-parsimony (MP) [7] methods in the program MEGA7 [19]. The NJ tree was constructed using Kimura's two-parameter model with complete deletion [17]. The ML tree was inferred using the nearest neighbor interchange as the maximum-likelihood heuristic search method. The MP tree was inferred using subtree-pruning and regrafting. The option of complete deletion of gaps was applied for ML and MP tree construction. A bootstrap analysis with 1000 replicate data sets was performed to assess support for clusters.

Chemotaxonomic Analyses

Cellular fatty acids of strain 15J8-9^T, *S. linguale* KACC 12156^T and *S. panaciterrae* KCTC 22263^T were analyzed using cells grown on R2A agar for 3 days at 25 °C. The cellular fatty acids were saponified, methylated, and extracted according to the Sherlock Microbial Identification System (MIDI) protocol. Fatty acid methyl esters were then analyzed by gas chromatography and Microbial Identification Software (Sherlock TSBA, version 6.0) [29]. Polar lipids were extracted using the procedure described by Minnikin et al. [27] and identified by two-dimensional thin layer chromatography (TLC), followed by spraying with the appropriate detection reagents [18]. The following spray reagents were used for detection: 5% molybdatophosphoric acid (Merck) in ethanol for total lipids; 0.2% ninhydrin in acetone for aminolipids; molybdenum blue (Sigma) for phospholipids; and α-naphthol-sulfuric acid reagent for glycolipids. Isoprenoid

quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under a vacuum, and re-extracted in n-hexane/water (1:1, v/v). The extract was purified using Sep-Pak Silica Vac cartridges (Waters) and then analyzed by high performance liquid chromatography (HPLC) as described previously [11].

Genomic Analysis

Genomic DNA of strain 15J8-9^T was extracted according to the standard CTAB/NaCl protocol [36]. Genomic DNA G + C content of strain 15J8-9^T was determined by reverse-phase HPLC analysis of individual nucleosides resulting from DNA hydrolysis and dephosphorylation using nuclease P1 and alkaline phosphatase [26]. Single-stranded DNA from salmon testes (D7656; Sigma; DNA G + C content, 41.2 mol%) was used as a standard.

DPD TaxonNumber and Nucleotide Sequence Accession Numbers

The Digital Protologue database TaxonNumber for strain 15J8-9^T is TA00195. The 16S rRNA gene sequence of strain 15J8-9^T obtained in this study has been deposited in NCBI GenBank/EMBL/DDBJ under the accession number LC221817. The accession numbers of the reference strains closely related to strain 15J8-9^T are indicated in Fig. 1.

Results and Discussion

Phylogenetic Analysis

A nearly complete 16S rRNA gene sequence of strain 15J8-9^T was a continuous stretch of 1416 bp. The sequence similarity search in the EzTaxon database revealed that this strain had highest similarity with *Spirosoma panaciterrae* Gsoil 1519^T (96.1%) [32], followed by *S. swuense* JBM2-3^T (94.9%) [13], and *S. aerolatum* PR1012K^T (94.0%) [15]. Levels of sequence similarity to other members of *Spirosoma* ranged from 93.2 to 90.9% and to other genera (*Fibrisoma, Huanghella, Rudanella*, and *Larkinella*) were less than 89%. The phylogenetic position of the new isolate, determined by various tree-making algorithms (NJ, ML, and MP), revealed that strain 15J8-9^T clustered with members of the genus *Spirosoma*, forming a subgroup with three above-mentioned *Spirosoma* species (Fig. 1). The generally accepted criteria for delineating bacterial species state that strains showing 16S rRNA gene sequence dissimilarity above 3% are considered to belong to a separate species [31, 34]. Based on this definition, the above data indicate that strain 15J8-9^T could not be assigned genetically to any recognized species within the genus *Spirosoma* and could be considered to represent a novel species of the genus *Spirosoma*. Based on phylogenetic analysis, its closest relative *S. panaciterrae* and the type species (*S. linguale*) of the genus *Spirosoma* were selected and used as reference strains in this study.

Morphological and Phenotypic Characteristics

Cells of strain 15J8-9^T were Gram-stain-negative, aerobic, non-motile, non-spore-forming, and yellow pigment-producing rods (0.9–1.1 μm wide and 3.5–5.2 μm long) without flagella (Supplementary Fig. S1). Cells were mesophilic, growing at 10–30 °C, but not at 4 or 37 °C, with an optimum temperature of around 25 °C. The isolate did not require NaCl for growth, but tolerated it up to 1%. Growth occurred on NA, R2A, and weakly on TSA agar, but not on LB agar. Other physiological and biochemical properties of strain 15J8-9^T are given in the species description. Phenotypic and chemotaxonomic characteristics that differentiated strain 15J8-9^T from its relatives in the genus *Spirosoma* are listed in Table 1. In particular, strain 15J8-9^T could be differentiated from *S. panaciterrae* KCTC 22263^T and *S. linguale* KACC 12156^T by its ability to grow at 10 °C, to produce *β*-glucuronidase and *α*-mannosidase, to utilize L-fucose and D-sorbitol, and to produce acid from D-sorbose.

Chemotaxonomic Characteristics

The major fatty acids of strain 15J8-9^T were summed feature 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*; 28.0%), $C_{16:1}$ *ω5c* (23.4%), iso-$C_{15:0}$ (13.5%), and $C_{16:0}$ (11.5%), representing 76.4% of the total fatty acids (Table 2). This cellular fatty acid profile is characteristic of members of the genus *Spirosoma* [6, 12, 15, 16], supporting an affiliation of strain 15J8-9^T with the genus *Spirosoma*. Under the same growth conditions, strain 15J8-9^T differed from *S. panaciterrae* KCTC 22263^T and *S. linguale* KACC 12156^T in terms of the proportions of major and some minor fatty acids. The major polar lipid found in strain 15J8-9^T was phosphatidylethanolamine (PE), similar to that in other *Spirosoma* species [1, 2, 8, 15]. In addition, the polar lipid profile of the isolate included moderate amounts of two unidentified aminophospholipids (APL_1 and APL_2) and an unidentified lipid (L_1), minor amounts of four unidentified lipids (L_2–L_5), and an unidentified aminolipid (AL) (Supplementary Fig. S2). The predominant isoprenoid quinone in strain 15J8-9^T was menaquinone MK-7, which is also the major respiratory quinone found in other members of the genus *Spirosoma* [1, 2, 4, 12, 21, 32].

Genomic Characteristics

The DNA G + C content of strain 15J8-9^T was 45.1 mol%, which is lower than values reported for *Spirosoma* species

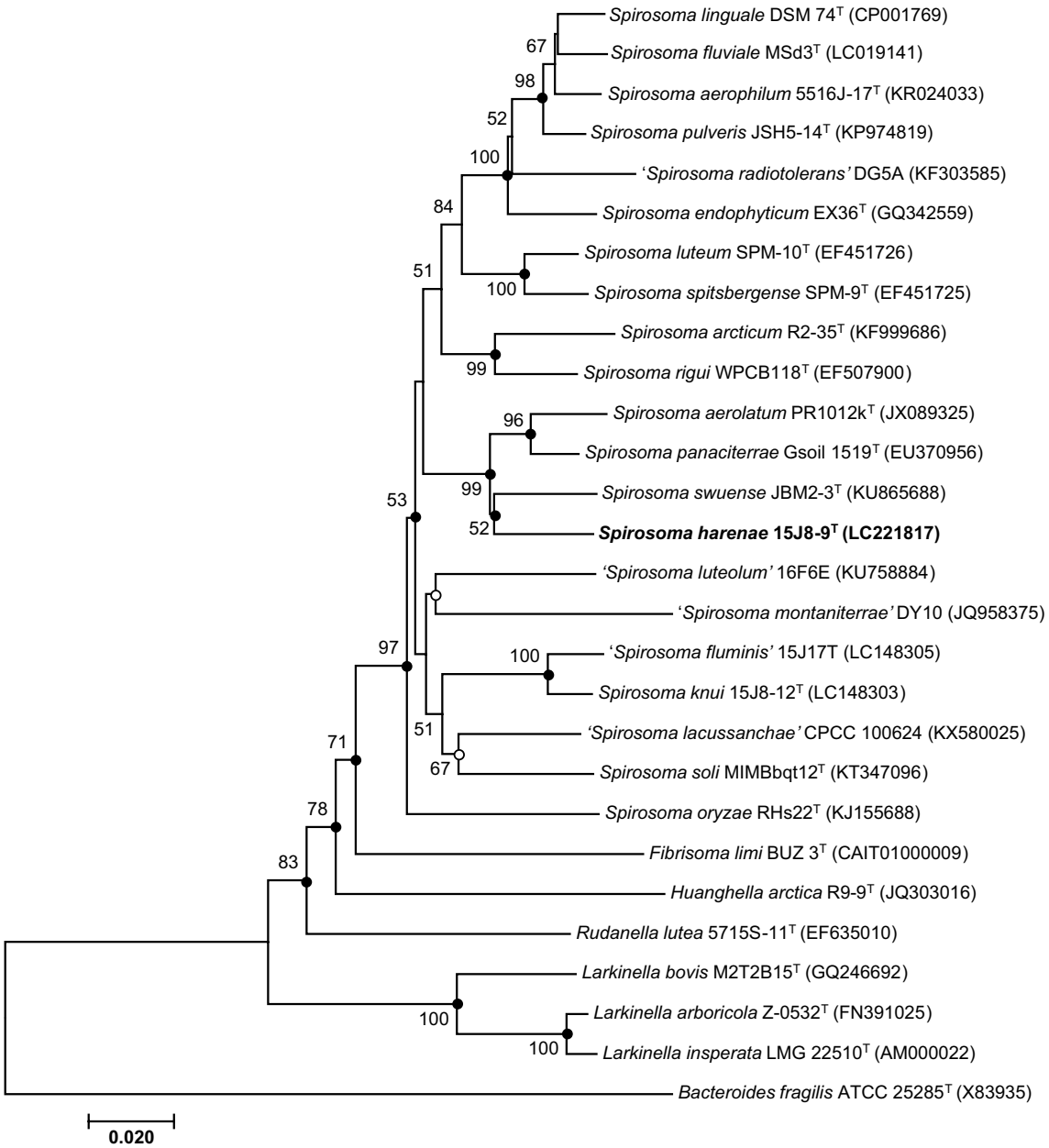

Fig. 1 Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the phylogenetic position of *Spirosoma harenae* 15J8-9^T among related strains in the genus *Spirosoma* and representatives of other members of phylum *Bacteroidetes*. Bootstrap values (based on 1000 replications) greater than 50% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. The tree was rooted using *Bacteroides fragilis* ATCC 25285^T (X83935) as an outgroup. Bar: 0.02 substitutions per nucleotide position

(47.2–57.0 mol%) [1, 8, 21]. However, the value still lies within the range expected for members of the same genus and the G + C content range of the genus *Spirosoma* should be extended to take into account this result.

Taxonomic Conclusion

The phenotypic and phylogenetic data presented here indicate that strain 15J8-9^T belongs to the genus *Spirosoma*.

Table 1 Differential phenotypic characteristics of strain 15J8-9^T and phylogenetically related species of the genus *Spirosoma*

Characteristic	1	2	3
Cell shape	Rods	Rods[a]	Rings, coils[b]
Growth at 10 °C	+	−	–
Growth at 37 °C	−	+	+
Indole production	−	−	+
Enzyme activity			
α-Chymotrypsin, cystine arylamidase	+	−	w
α-Fucosidase	−	w	−
β-Galactosidase	+	−	w
β-Glucuronidase	w	−	−
α-Mannosidase	+	−	−
Trypsin, valine arylamidase	+	−	+
Assimilation of			
N-Acetyl-glucosamine	w	+	−
L-Arabinose	−	+	−
L-Fucose, D-sorbitol	w	−	−
3-Hydroxybenzoate, itaconate	−	−	w
L-Rhamnose	w	−	w
D-Sucrose	+	+	−
Acid production from			
Amygdalin, glycogen, starch, D-tagatose	w	−	+
D-Arabinose, L-arabinose, arbutin, D-fructose, D-fucose, gentiobiose, D-lactose, D-melibiose, D-melezitose, methyl-*α*-D-glucopyranoside, methyl-*α*-D-mannopyranoside, D-raffinose, D-sucrose, D-xylose	+	−	+
D-Arabitol, L-arabitol, 5-ketogluconate, xylitol	−	−	+
L-Fucose, D-ribose	w	−	w
Gluconate, inulin, 2-ketogluconate	−	−	w
D-Lyxose, L-rhamnose, D-turanose	+	−	w
Methyl-*β*-D-xylopyranoside, L-xylose	w	−	w
D-Sorbitol	−	+	−
D-Sorbose	w	−	−
DNA G+C content (mol%)	45.1	50.1[a]	50.2[b]

All data were obtained in this study, unless otherwise noted

(+) positive reaction, (–) negative reaction, (w) weakly positive reaction

Strains: 1, 15J8-9^T; 2, *S. panaciterrae* KCTC 22263^T; 3, *S. linguale* KACC 12156^T

All strains grew on NA, R2A, and TSA agar, but not on LB agar. All strains were positive for *N*-acetyl-*β*-glucosaminidase, acid phosphatase, alkaline phosphatase, catalase, esterase (C4), esterase lipase (C8), *β*-galactosidase, *α*-glucosidase, *β*-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and oxidase activities. All species were found negative for nitrite reduction, gelatin hydrolysis, arginine dihydrolase, *α*-fucosidase, lipase (C14), and urease activities. All strains produced acid from *N*-acetyl-glucosamine, aesculin, D-cellobiose, D-galactose, D-maltose, D-mannose, salicin, and D-trehalose, but not from D-adonitol, dulcitol, erythritol, D-glucose, glycerol, inositol, or D-mannitol. All strains utilized D-glucose, D-maltose, D-mannose, D-melibiose, and salicin, but not acetate, adipate, L-alanine, caprate, citrate, gluconate, glycogen, L-histidine, 4-hydroxybenzoate, DL-3-hydroxybutyrate, m-inositol, 2-ketogluconate, 5-ketogluconate, DL-lactate, L-malate, malonate, D-mannitol, phenyl acetate, L-proline, propionate, D-ribose, L-serine, suberate, or n-valerate

[a]Data from Reference [32]

[b]Data from Reference [20]

Phylogenetic distinctiveness confirmed that this isolate represents a species that is distinct from other members of the genus *Spirosoma*. Strain 15J8-9^T can be differentiated from the phylogenetically closest relative *S. panaciterrae* and the type species of the genus *Spirosoma, S. linguale*, based on several phenotypic characteristics (Table 1). Therefore, on the basis of the data presented, strain 15J8-9^T is considered to represent a novel species of the genus

Table 2 Cellular fatty acid profiles of strain 15J8-9^T and phylogenetically related members of the genus *Spirosoma*

Fatty acids	1	2	3
Saturated			
Iso-$C_{13:0}$	2.4	nd	2.2
$C_{14:0}$	1.3	2.0	tr
Iso-$C_{15:0}$	13.5	5.9	14.4
Anteiso-$C_{15:0}$	2.9	tr	4.6
Iso-$C_{15:0}$ 3-OH	2.1	2.7	3.2
$C_{16:0}$	11.5	14.3	4.5
$C_{16:0}$ 3-OH	1.5	3.7	tr
Iso-$C_{17:0}$	tr	nd	1.3
Iso-$C_{17:0}$ 3-OH	7.2	3.1	5.3
$C_{18:0}$	4.3	9.2	tr
Unsaturated			
$C_{16:1}$ *ω5c*	23.4	24.6	24.9
Summed feature 3[a]	28.0	30.6	28.7
Summed feature 4[a]	tr	nd	1.2
Summed feature 9[a]	tr	nd	2.5

All data are from the present study. All strains were grown on R2A agar at 25 °C for 3 days. Values are percentages of total fatty acids, and only fatty acids accounting for more than 1% in at least one of the strains are indicated

nd not detected, *tr* trace (< 1.0%)

Strains: 1, 15J8-9^T; 2, *S. panaciterrae* KCTC 22263^T; 3, *S. linguale* KACC 12156^T

[a]Summed feature contained two or three fatty acids that could not be separated by gas–liquid chromatography (GLC) with the Sherlock Microbial Identification (MIDI) System. Summed feature 3 comprised $C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c*. Summed feature 4 comprised iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B. Summed feature 9 comprised iso-$C_{17:1}$ *ω9c* and/or $C_{16:0}$ 10-methyl

Spirosoma, for which the name *Spirosoma harenae* sp. nov. is proposed.

Description of *Spirosoma harenae* sp. nov

Spirosoma harenae (ha.re'nae. L. gen. n. *harenae* of sand, from where the organism was isolated).

Cells are Gram-stain-negative, non-motile, aerobic rods that are 0.9–1.1 μm wide and 3.5–5.2 μm long. After 3 days of incubation at 25 °C on R2A agar, colonies are convex, smooth, circular, yellow and approximately 1 mm in diameter. Growth occurs at 10–30 °C and pH 5–8, with optimal growth at 25 °C and pH 7. Tolerate 1% of NaCl but not 2%. Cells are positive for catalase and oxidase activities. In API 20 NE tests, positive for aesculin hydrolysis, but negative for gelatin hydrolysis, nitrate reduction, indole production, acid production from D-glucose, and urease and arginine dihydrolase activity. Utilizes *N*-acetyl-D-glucosamine (weakly, w), L-fucose, D-glucose, D-maltose, D-mannose, D-melibiose, L-rhamnose (w), salicin, D-sorbitol (w), and D-sucrose, but not other substrates in API 32 GN and API 20 NE kits (Supplementary Table S1). In API ZYM tests, positive for *N*-acetyl-*β*-glucosaminidase, acid phosphatase, alkaline phosphatase, *α*-chymotrypsin, cysteine arylamidase, esterase (C4) (w), esterase lipase (C8), *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, *β*-glucosidase, *β*-glucuronidase (w), leucine arylamidase, *α*-mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin, and valine arylamidase; negative for other enzyme activities. Acid is produced from *N*-acetyl-glucosamine (weakly, w), aesculin, amygdalin (w), L-arabinose, D-arabinose, arbutin, D-cellobiose, D-fructose, D-fucose, L-fucose, D-galactose, gentiobiose, glycogen (w), D-lactose, D-lyxose, D-maltose, D-mannose, D-melibiose, D-melezitose, methyl-*α*-D-glucopyranoside, methyl-*α*-D-mannopyranoside, methyl-*β*-D-xylopyranoside (w), D-raffinose, L-rhamnose, D-ribose (w), salicin, L-sorbose, starch, D-sucrose, D-tagatose (w), D-trehalose, D-turanose, D-xylose, and L-xylose, but not from other substrates tested in the API 50CH system. The major cellular fatty acids are summed feature 3 ($C_{16:1}$ *ω7c*/$C_{16:1}$ *ω6c*), $C_{16:1}$ *ω5c*, iso $C_{15:0}$, and $C_{16:0}$. The predominant isoprenoid quinone is MK-7. Phosphatidylethanolamine is the major polar lipid. The DNA G+C content is 45.1 mol%. The type strain 15J8-9^T (= KCTC 52030^T = JCM 31993^T) was isolated from beach sand sample collected in Jeju Island (29°51′61″ N, 126°27′08″ E), South Korea.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflict of interest.

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