Spirosoma knui sp. nov., a radiation-resistant bacterium isolated from the Han River

Jae-Jin Lee, Yeon-Hee Lee, Su-Jin Park, Seung-Yeol Lee, Byung-Oh Kim, Leonid N. Ten, Myung Kyum Kim and Hee-Young Jung

Abstract

A Gram-stain-negative, non-motile, non-spore-forming, rod-shaped, aerobic bacterium, designated 15J8-12\(^T\), was isolated from a water sample after exposure to 3 kGy of gamma radiation. The strain showed resistance to gamma radiation with a dose required to reduce the bacterial population 10 fold (D\(_{10}\)) value of 4.7 kGy. The results of comparative 16S rRNA gene sequence analysis indicated that strain 15J8-12\(^T\) represented a member of the family Cytophagaceae, phylum Bacteroidetes, and was most closely related to ‘Spirosoma fluminis’ 15J17 (97.92 %) and Spirosoma arcticum R2-35\(^T\) (92.22 %). The G+C content of the genomic DNA of 15J8-12\(^T\) was 51.3 mol%. The detection of menaquinone MK-7 as the predominant respiratory quinone, a fatty acid profile with summed feature 3 (C\(_{16:1}\)ω7c/C\(_{16:1}\)ω6c; 40.5 %), C\(_{16:1}\)ω5c (35.3 %), C\(_{15:0}\) iso (6.9 %) and C\(_{16:0}\) (6.8 %) as the major components and phosphatidylethanolamine as the major polar lipid also supported the affiliation of 15J8-12\(^T\) with the genus Spirosoma. The DNA–DNA relatedness between 15J8-12\(^T\) and ‘Spirosoma fluminis’ 15J17 was 27.8 %. On the basis of its phenotypic and genotypic properties, together with its phylogenetic distinctiveness, 15J8-12\(^T\) should be considered to be a representative of a novel species of the genus Spirosoma, for which the name Spirosoma knui sp. nov. is proposed. The type strain is 15J8-12\(^T\) (=KCTC 52510\(^T\)=JCM 31407\(^T\)).

The genus Spirosoma was first reported by Larkin and Borrall [1], and this description was later emended by Finster et al. [2] and Ahn et al. [3]. Members of the genus Spirosoma are Gram-negative, 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\(_{16:1}\)ω7c/C\(_{16:1}\)ω6c; 40.5 %), C\(_{16:1}\)ω5c (35.3 %), C\(_{15:0}\) iso (6.9 %) and C\(_{16:0}\) (6.8 %) as the major fatty acids. At the time of writing, the genus Spirosoma comprised ten species with validly published names, with Spirosoma lin- guale as the type species. However, several other strains, such as Spirosoma aerophilum 55161-17\(^T\) [4], Spirosoma fluminis’ 15J17 [5], Spirosoma soli MIMBqt12\(^T\) [6], and Spirosoma swuense JB2M-3\(^T\) [7] have recently been proposed as novel species of the genus Spirosoma. The type strains of species of the genus Spirosoma have been recovered from soil, fresh water, plant xylem sap and extreme environments, such as high Arctic glacial till or Arctic permafrost soil [2, 3, 8]. Moreover, some members of the genus Spirosoma have been reported to be resistant to ionizing radiation [7, 9]. In the present report, we describe a novel radiation-resistant strain, designated 15J8-12\(^T\), which was isolated from a water sample that was preliminarily irradiated with gamma rays. To determine the precise taxonomic position of 15J8-12\(^T\), a phylogenetic analysis based on 16S rRNA gene sequences, DNA–DNA hybridization and an analysis of the phenotypic, genotypic and chemotaxonomic characteristics of the isolate were performed. On the basis of the results obtained in this study, we propose that 15J8-12\(^T\) should be placed in the genus Spirosoma as the type strain of a novel species.

Strain 15J8-12\(^T\) was isolated from water collected from the Han River, South Korea (37° 31' 40" N, 126° 56' 06" E). The water sample was filtered through a membrane filter ( pore size, 0.2 µm; Millipore), the filter was vortexed in 10 ml of distilled water, and then the aqueous sample was exposed to 3 kGy of gamma radiation using a cobalt-60 irradiator (MDS Nordion). A 1 ml aliquot of irradiated sample was serially diluted, and 100 µl of each dilution was spread on R2A agar and incubated at 25 °C for 1 week. Single colonies on the plates were purified by transfer onto fresh plates and incubation again under the same conditions. Isolate 15J8-
12<sup>T</sup> was routinely cultured on R2A agar at 25 °C and was maintained as a glycerol suspension (20 %, w/v) at −70 °C. *Spirosoma arcticum* KACC 18577<sup>T</sup> and *Spirosoma linguale* KACC 12156<sup>T</sup> were obtained from the Korean Agricultural Culture Collection and used as reference strains.

For the phylogenetic analysis, the 16S rRNA gene was amplified from chromosomal DNA using the universal bacterial primers 9F and 1512R as described previously [10], and purified PCR products were sequenced by Genotech (Deajeon, South Korea). The nearly complete 16S rRNA gene sequences were assembled using SeqMan software (DNASTAR). Phylogenetic neighbours were identified, and pairwise 16S rRNA gene sequence similarities were calculated using both the EzTaxon-e server [11] and NCBI BLAST searches. The 16S rRNA gene sequences of related taxa were obtained from GenBank and aligned with that of 15J8-12<sup>T</sup> using the program CLUSTAL X [12]. Gaps and the 5' and 3' ends of the alignment were edited manually in BioEdit [13]. Tree topologies were inferred by neighbour-joining [14], maximum-likelihood (ML) [15], and maximum-parsimony (MP) [16] methods in the program MEGA7 [17]. The neighbour-joining tree was reconstructed using Kimura's two-parameter model with pairwise deletion [18]. The ML tree was inferred using the nearest neighbour 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 reconstruction. A bootstrap analysis with 1000 replicate data sets was performed to assess support for clusters [19].

A nearly complete 16S rRNA gene sequence of 15J8-12<sup>T</sup> (1444 bp) was obtained. Based on 16S rRNA gene sequence similarities, the closest relatives of 15J8-12<sup>T</sup> using the program CLUSTAL X [12]. Gaps and the 5' and 3' ends of the alignment were edited manually in BioEdit [13]. Tree topologies were inferred by neighbour-joining [14], maximum-likelihood (ML) [15], and maximum-parsimony (MP) [16] methods in the program MEGA7 [17]. The neighbour-joining tree was reconstructed using Kimura's two-parameter model with pairwise deletion [18]. The ML tree was inferred using the nearest neighbour 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 reconstruction. A bootstrap analysis with 1000 replicate data sets was performed to assess support for clusters [19].

Gram reaction of 15J8-12<sup>T</sup> was examined using a staining method [22]. The morphology and motility of cells of 15J8-12<sup>T</sup>, grown for 3 days at 25 °C on R2A agar, were observed under a light microscope (Olympus; ×1000 magnification) and a HIT7700 transmission electron microscope (Hitachi). Catalase activity was determined by assessing the production of bubbles in 3 % (v/v) H<sub>2</sub>O<sub>2</sub>, and oxidase activity was tested using 1 % (w/v) tetramethyl-p-phenylenediamine [23]. 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 after incubation for 7 days on R2A agar. Salt tolerance was tested in R2A broth supplemented with 0.5, 1, 2, 3, 4, 5 or 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).

Cells of 15J8-12<sup>T</sup> were rod-shaped and had a tendency to form short filaments (Fig. S2). Colonies on R2A plates after 3 days at 25 °C were flat to convex, translucent, circular, yellowish and 1–2 mm in diameter. Cells were mesophilic, growing at 10–37 °C but not at 4 or 42 °C, with an optimum temperature of around 25 °C. This strain grew at pH 5–9, with optimal growth at pH 7. Growth occurred on R2A agar, nutrient agar (NA), trypticase soy agar (TSA) and weakly on Luria-Bertani (LB) agar. The isolate tolerated 1 % but not 2 % (w/v) NaCl. In common with other strains of species of the genus *Spirosoma* with validly published names [3, 8, 24, 25], 15J8-12<sup>T</sup> was negative for Gram reaction, nitrate reduction, arginine dihydrolase, glucose fermentation and assimilation of adipate, L-arabinose, caprate, citrate, L-malate and phenylacetate but positive for alkaline phosphatase, catalase, leucine arylamidase and aesculin hydrolysis. Phenotypic and chemotaxonomic characteristics differentiating 15J8-12<sup>T</sup> from its closest relatives in the genus *Spirosoma* are listed in Table 1.

Genomic DNA of 15J8-12<sup>T</sup> and the most closely related strain, *S. fluminis* 15J17, were extracted according to the standard CTAB/NaCl protocol [26]. Genomic DNA G+C content of 15J8-12<sup>T</sup> was determined by a reverse-phase HPLC analysis of individual nucleosides resulting from DNA hydrolysis and dephosphorylation using nuclease P1 and alkaline phosphatase [27]. Single-stranded DNA from salmon testes (D7656; Sigma; DNA G+C content, 41.2 mol%) was used as a standard. Isopenoid 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 analyzed by HPLC as described previously [28]. Cellular fatty acids were analysed using cells grown on R2A agar for 3 days at 25 °C. Cellular fatty acid saponification, extraction and
methylation were performed according to the Sherlock Microbial Identification System (MIDI) protocol. Fatty acid methyl esters were then analyzed by gas chromatography using the Microbial Identification software package (TSBA, version 6.0) [29]. Polar lipids were extracted using a procedure described by Minnikin et al.
and identified by two-dimensional thin layer chromatography, followed by spraying with the appropriate detection reagents [31].

The DNA G+C content of 15J8-12T was 51.3 mol%, which lies within the range observed for members of the genus Spirosoma with validly published names [3, 25, 32, 33].
fatty acid profile of 15J8-12<sup>T</sup> was characterized by the presence of summed feature 3 (C<sub>16:1ω7c/C<sub>16:1ω6c; 40.5%)), C<sub>16:1ω5c; 35.3%), C<sub>15:0 iso (6.9%) and C<sub>16:0 (6.8%) as major fatty acids. This profile is similar to those of the phylogenetically closest strains ‘S. fluminis’ 15J17, S. arcticum KACC 18577<sup>T</sup> and S. linguale KACC 12156<sup>T</sup> (Table 2) and those of other members of the genus *Spirosoma* [3, 5]. However, certain qualitative and quantitative differences in fatty acid content were observed between 15J8-12<sup>T</sup> and its closest relatives. Specifically, 15J8-12<sup>T</sup> could be differentiated from the above mentioned species by its higher content of summed feature 3 and C<sub>16:1ω5c</sub>, its lower amount of C<sub>17:0 iso 3-OH</sub>, the presence of a small quantity of C<sub>13:1</sub> at 12–13 and the absence of C<sub>15:0 iso 3-OH, C<sub>16:0 3-OH</sub>, and summed features 4 and 9. The major polar lipid in 15J8-12<sup>T</sup> was phosphatidylethanolamine (PE), as in other species of the genus *Spirosoma* [6, 24]. In addition, the polar lipid profile of the isolate included moderate amounts of an unknown aminolipid AL<sub>3</sub>, an unknown aminophospholipid (APL), and lipids L<sub>1</sub> and L<sub>10</sub>, and minor quantities of two unknown aminolipids (AL<sub>1</sub> and AL<sub>2</sub>), three unknown glycolipids (GL<sub>1</sub>–GL<sub>3</sub>), two unknown phospholipids (PL<sub>1</sub> and PL<sub>2</sub>) and nine unknown polar lipids (L<sub>1</sub>–L<sub>3</sub>, L<sub>5</sub>–L<sub>9</sub> and L<sub>11</sub>) (Fig. S3). The predominant isoprenoid quinone in 15J8-12<sup>T</sup> was menaquinone MK-7, which is also the major respiratory quinone found in other members of the genus *Spirosoma* [4, 34].

Survival of 15J8-12<sup>T</sup> after exposure to various doses of gamma radiation was evaluated as described previously [35]. Cells of *Deinococcus radiodurans* DSM 20539<sup>T</sup> and *Escherichia coli* KCTC 1116 were used as positive and negative controls, respectively. *E. coli* KCTC 1116 did not grow after a gamma radiation dose of 2.0 kGy. In contrast, 15J8-12<sup>T</sup> appeared to be somewhat resistant to ionizing radiation, but significantly less so than *D. radiodurans* DSM 20539<sup>T</sup>. Exposure of these two cultures to 2 and 4 kGy of gamma radiation resulted in 50 and 30 % cell survival, respectively, for 15J8-12<sup>T</sup>, compared with 95 and 90 % for *D. radiodurans* DSM 20539<sup>T</sup>. 15J8-12<sup>T</sup> tolerated high doses of gamma radiation with a D<sub>10</sub> (the dose required to reduce the bacterial population 10 fold) of 4.7 kGy, which was approximately two times less than that reported for radiation resistant ‘*Spirosoma radiotolerans*’ DG5A [9], demonstrating a greater sensitivity to such stress.

DNA–DNA hybridization was performed fluorometrically using the method of Ezaki et al. [36], with photobiotin-labeled DNA probes and microdilution wells. The highest

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**Table 2.** Cellular fatty acid profiles of 15J8-12<sup>T</sup> and its phylogenetically closest relatives in the genus *Spirosoma*

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td><strong>Saturated</strong></td>
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</tr>
<tr>
<td>C&lt;sub&gt;13:0 iso&lt;/sub&gt;</td>
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<td>–</td>
<td>TR</td>
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<tr>
<td>C&lt;sub&gt;14:0&lt;/sub&gt;</td>
<td>1.3</td>
<td>2.4</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;15:0 iso&lt;/sub&gt;</td>
<td>6.9</td>
<td>6.7</td>
<td>7.2</td>
<td>14.4</td>
</tr>
<tr>
<td>C&lt;sub&gt;15:0 anteiso&lt;/sub&gt;</td>
<td>2.6</td>
<td>1.9</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;15:0 iso 3-OH&lt;/sub&gt;</td>
<td>–</td>
<td>1.9</td>
<td>4.3</td>
<td>3.2</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>6.8</td>
<td>13.1</td>
<td>8.6</td>
<td>4.5</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:0 3-OH&lt;/sub&gt;</td>
<td>–</td>
<td>1.5</td>
<td>2.5</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:0 N alcohol&lt;/sub&gt;</td>
<td>–</td>
<td>–</td>
<td>12.2</td>
<td>–</td>
</tr>
<tr>
<td>C&lt;sub&gt;17:0 iso&lt;/sub&gt;</td>
<td>–</td>
<td>–</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>C&lt;sub&gt;17:0 iso 2-OH&lt;/sub&gt;</td>
<td>–</td>
<td>–</td>
<td>1.2</td>
<td>TR</td>
</tr>
<tr>
<td>C&lt;sub&gt;17:0 iso 3-OH&lt;/sub&gt;</td>
<td>1.0</td>
<td>4.8</td>
<td>11.5</td>
<td>5.3</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:0&lt;/sub&gt;</td>
<td>2.0</td>
<td>8.1</td>
<td>TR</td>
<td>TR</td>
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<td><strong>Unsaturated</strong></td>
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<tr>
<td>C&lt;sub&gt;13:1&lt;/sub&gt; at 12–13</td>
<td>2.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1ω5c&lt;/sub&gt;</td>
<td>35.3</td>
<td>22.3</td>
<td>11.5</td>
<td>24.9</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1ω9c&lt;/sub&gt;</td>
<td>–</td>
<td>1.1</td>
<td>–</td>
<td>TR</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>40.5</td>
<td>30.8</td>
<td>26.3</td>
<td>28.7</td>
</tr>
<tr>
<td>Summed feature 4*</td>
<td>–</td>
<td>TR</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Summed feature 9*</td>
<td>–</td>
<td>2.0</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

*Summed feature contained two fatty acids that could not be separated by gas–liquid chromatography (GLC) with the Sherlock Microbial Identification (MIDI) System. Summed feature 3 comprised C<sub>16:1ω7c</sub> and/or C<sub>16:1ω6c</sub>; summed feature 4 comprised C<sub>17:1 iso 1</sub> and/or C<sub>17:1 anteiso B</sub>; and summed feature 9 comprised C<sub>17:1 iso ω9c</sub> and/or C<sub>16:0 10-methyl</sub>. C<sub>13:1</sub> at 12–13 is tridecenoic acid that contains double bond between carbons 12 and 13.
and lowest values from five replications for each sample were excluded, and the means of the remaining three values were recorded as the DNA–DNA hybridization values. DNA from 15J8–12T showed relatively low DNA–DNA relatedness with that of ‘S. fluminis’ 15J17, with a value (27.8%) significantly below the recommended cut-off threshold of 70% for the identification of bacterial species [20]. This result indicated that the two strains differed from each other at the species level.

Phenotypic and phylogenetic characteristics of 15J8–12T indicated that it represented a member of the genus Spirosoma. However, there were several phenotypic differences between 15J8–12T and its phylogenetically closest relatives (Table 1). Thus, results of phenotypic and genotypic analyses indicated that 15J8–12T satisfied all of the previously described requirements for documenting that an isolate represents a novel species. Therefore, based on the data presented, strain 15J8–12T should be classified as a representative of a novel species of the genus Spirosoma, for which the name Spirosoma knui sp. nov. is proposed.

DESCRIPTION OF SPIROSOMA KNUI SP. NOV.

Spirosoma knui sp. nov. (knui‘i. N.L. gen. n. knui of or belonging to KNU, Kyungpook National University, where the taxonomic study was performed).

Cells are Gram-stain-negative, non-motile, aerobic rods, 0.8–1.0 µm wide and 1.1–6.5 µm long. After 3 days of incubation at 25 °C on R2A agar, colonies are flat to convex, translucent, circular, yellowish and slimy. Cells are positive for catalase and oxidase activities. In API 20 NE tests, positive for acetic acid hydrolysis and β-galactosidase but negative for gelatin hydrolysis, nitrate reduction, indole production, glucose fermentation, urea hydrolysis and arginine dihydrolase activity. In API ZYM test, esterase lipase (C8) (weakly), esterase lipase (2-ketogluconatase, 5-ketogluconatase, D-mannitol, D-melezitose, methyl β-D-xylopyranoside, D-ribose, D-sorbitol, L-sorbose, D-tagatose, xylitol, or D-xylene. The major cellular fatty acids are summed feature 3 (C16:1ω7c/C16:1ω6c, C16:1ω5c, C15:0 iso and C16:0). The predominant isoprenoid quinone is MK-7. Phosphatidylethanolamine is the major polar lipid. The DNA G+C content of the type strain is 51.3 mol%.

The type strain 15J8–12T (=KCTC 52510T=JCM 31407T) was isolated from a water sample collected from the Han River (37°31’40”N, 126°56’06”E), South Korea.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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