Spirosoma fluviale sp. nov., isolated from river water

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A bacterial strain, designated MSd3^T, was isolated from a freshwater sample collected from the Hosoda River in Japan. The cells of strain MSd3^T were Gram-stain-negative, non-spore-forming, aerobic, non-motile, curved rods forming rings, coils and undulating filaments. The 16S rRNA gene sequence of strain MSd3^T showed closest similarity to that of *Spirosoma linguale* DSM 74^T (97.6 % similarity) and similarity to other members of the genus *Spirosoma* ranged from 90.3 to 95.9 %. Strain MSd3^T contained menaquinone 7 as the sole respiratory quinone. The major cellular fatty acids were summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) and $C_{16:1}\omega5c$. The polar lipids were phosphatidylethanolamine, three unidentified aminophospholipids and three unidentified polar lipids. The DNA G+C content was 53.3 mol%. The DNA–DNA relatedness between strain MSd3^T and *S. linguale* DSM 74^T was 19 % or 25 % (reciprocal value). From the chemotaxonomic and physiological data and the levels of DNA–DNA relatedness, strain MSd3^T should be classified as the representative of a novel species of the genus *Spirosoma*, for which the name *Spirosoma fluviale* sp. nov. (type strain MSd3^T=JCM 30659^T=DSM 29961^T) is proposed.

The genus *Spirosoma*, which was first described by Migula in 1894 (Migula, 1894), was included in the Approved Lists of Bacterial Names by Skerman *et al.* (1980). The description of this genus (Larkin & Borrall, 1984) was emended by Finster *et al.* (2009) and Ahn *et al.* (2014). Cells of members of this genus are Gram-stain-negative rods often resulting in rings, coils and undulating filaments, and produce yellow or orange, diffusible or non-diffusible pigments that do not belong to the flexirubin type. Chemotaxonomic characteristics of the genus include the following: the major respiratory quinone is menaquinone 7 (MK-7); the major cellular fatty acids are summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$), $C_{16:1}\omega5c$, iso-$C_{15:0}$ and $C_{16:0}$; DNA G+C content ranges from 47.2 to 57.0 mol%. The genus *Spirosoma* comprised eight species with validly published names at the time of writing. Strains of this genus were isolated from soil, fresh water, *Salix caprea* trees (Fries *et al.*, 2013) and a glacier till (Chang *et al.*, 2014).

During the isolation of various pigment-producing bacteria from environmental samples, we isolated strain MSd3^T from a freshwater sample collected from the Hosoda River in Japan. Strain MSd3^T was cultivated from the sample on an IPM plate [l^{-1} : 0.5 g yeast extract, 0.5 g proteose peptone (Difco no. 3), 0.5 g tryptone, 0.5 g sucrose, 0.3 g sodium pyruvate, 0.3 g K_2HPO_4, 0.3 g $MgSO_4.7H_2O$, 0.3 g $CaCl_2.2H_2O$, 15 g Gellan gum, pH 6.6] aerobically at 25 °C. The strain formed chrome-yellow colonies on IPM plates. Strain MSd3^T also grew aerobically on R2A agar (Reasoner & Geldreich, 1985). This strain was routinely cultured on R2A agar or in liquid R2A medium. In this study, the taxonomic position of this strain was investigated.

Genomic DNA was extracted from strain MSd3^T using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). PCR amplification of the 16S rRNA gene and sequencing were performed as previously described (Hatayama *et al.*, 2005). Similarity-based search was performed using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). The 16S rRNA gene sequence of strain MSd3^T (nucleotide positions 25–1415) was aligned with those of the type strains of related genera using the CLUSTAL X software package (Thompson *et al.*, 1997). Evolutionary distances were calculated using the Kimura two-parameter method (Kimura, 1980), and were then used by the MEGA6 program (Tamura *et al.*, 2013) to reconstruct a phylogenetic tree through the neighbour-joining method (Saitou & Nei, 1987) with bootstrap values

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Spirosoma fluviale* MSd3^T is LC019141.

One supplementary table and one supplementary figure are available with the online Supplementary Material.

(Felsenstein, 1985) based on 1000 replications. Other phylogenetic trees were reconstructed by using the maximum-likelihood method (Tamura & Nei, 1993) and the maximum-parsimony method (Nei & Kumar, 2000) in the MEGA6 program, with bootstrap values based on 1000 replications.

The 16S rRNA gene sequence of strain MSd3^T showed closest similarity to that of *Spirosoma linguale* DSM 74^T (97.6 % similarity) and similarities to those of other members of the genus *Spirosoma* (90.3–95.9 %). Phylogenetic analyses based on the 16S rRNA gene sequences performed by using the neighbour-joining, maximum-likelihood and maximum-parsimony methods indicated that strain MSd3^T belonged to the genus *Spirosoma* and its closest phylogenetic neighbour was *S. linguale* (Fig. 1).

Physiological characteristics of strain MSd3^T were determined and compared with those of *S. linguale* DSM 74^T as a reference strain. The temperature range for growth was tested on R2A agar at 4, 20, 25, 30, 37 and 42 °C. The pH range for growth was determined in liquid R2A medium that was adjusted to pH 3.0–11.0 (in increments of 1 pH unit) using 50 mM citrate buffer (pH 3.0, 4.0), 50 mM MES (pH 5.0, 6.0), 50 mM HEPES (pH 7.0, 8.0), 50 mM CHES (pH 9.0, 10.0) and 50 mM CAPS (pH 11.0). Anaerobic growth was tested on R2A agar by using the Anaero Pack System (Mitsubishi Gas Chemical). Cell morphology and motility were observed during the lag, exponential and stationary phases of growth under a phase-contrast microscope. Gliding motility was investigated by the method of Finster *et al.* (2009). The Gram reaction of cells was performed by the traditional staining method and the nonstaining (KOH) method (Buck, 1982). Flexirubin-type pigment production was determined as described by Bernardet *et al.* (2002). Utilization of carbon sources was determined by using the API 20 E and API 20 NE (bioMérieux) and the Biolog GN2 (Biolog) systems. Some physiological characteristics were determined with the API 20 E and API 20 NE systems. Young cultures (22 h old) grown on R2A agar at 30 °C were inoculated to these systems. The results of these systems were evaluated after 48 h of incubation at 30 °C. Catalase and oxidase activities were tested according to previously described methods (Hatayama, 2014). NaCl tolerance was studied using R2A agar supplemented with 1–5 % (w/v) NaCl. Resistance to antibiotics was determined by adding antibiotics (ampicillin, chloramphenicol, kanamycin, rifampicin, streptomycin, polymyxin B and tetracycline) from filter-sterilized stock solutions to R2A liquid medium to final concentrations of 10 μg ml^{-1}. The physiological characteristics of strain MSd3^T are summarized in Table 1 and in the species description. Different physiological characteristics between strain MSd3^T and *S. linguale* DSM 74^T are summarized in Table 1. Gliding motilities of strain MSd3^T and *S. linguale* DSM 74^T were not observed in this study.

Profiles of respiratory quinones and cellular fatty acids were determined by TechnoSuruga Laboratory (Shizuoka, Japan). Extraction of respiratory quinones was performed by the method of Nishijima *et al.* (1997) prior to analysis by HPLC. Cellular fatty acids were identified using the Sherlock Microbial Identification System (version 6) (MIDI) with the TSBA6 library. Cells grown aerobically in liquid R2A medium for 1 day at 30 °C (corresponding to the mid- to late-exponential growth phase) were used for analyses

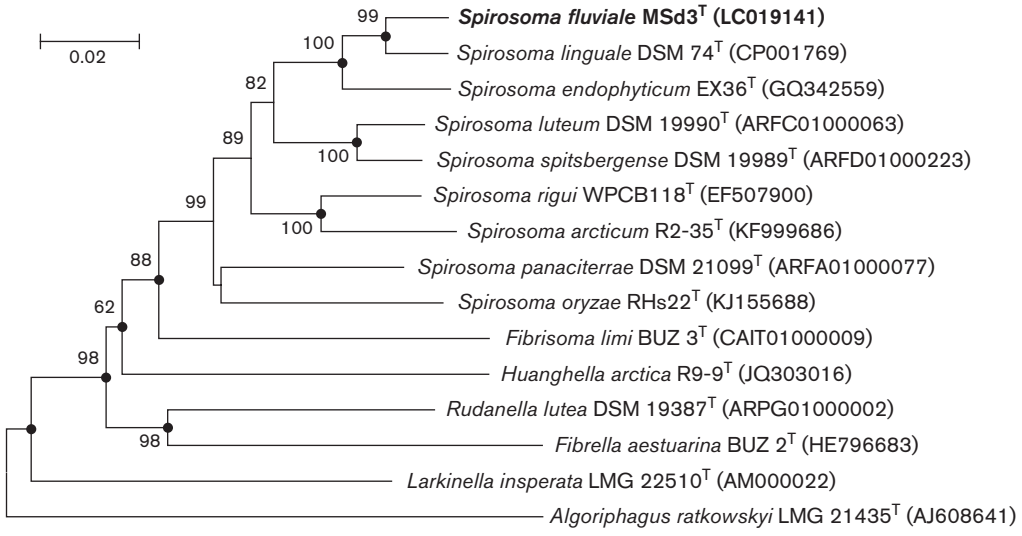

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain MSd3^T and type strains of species of related genera. The tree is drawn using the neighbour-joining method. Bar, 0.02 substitutions per nucleotide position. Bootstrap values are calculated from 1000 repeats and those greater than 50 % are shown at branch points. Numbers in parentheses are the accession numbers of the GenBank/EMBL/DDBJ database. Filled circles indicate that the corresponding nodes were also recovered in trees drawn using the maximum-likelihood method and the maximum-parsimony method.

Table 1. Differential characteristics between strain MSd3^T and *S. linguale* DSM 74^T

DNA G+C content for *S. linguale* DSM 74^T was obtained from Larkin & Borrall (1984); the remaining data were obtained in this study. +, Positive; −, negative.

Characteristic	MSd3^T	DSM 74^T
Growth at 37 °C	−	+
Resistance to kanamycin (10 μg ml^{-1})	−	+
Oxidization of (Biolog GN2):		
Dextrin	−	+
N-Acetyl-D-galactosamine	−	+
D-Arabitol	+	−
L-Fucose	−	+
L-Rhamnose	−	+
α-Hydroxybutyric acid	−	+
γ-Hydroxybutyric acid	−	+
α-Ketobutyric acid	−	+
Succinic acid	−	+
Bromosuccinic acid	−	+
L-Alaninamide	−	+
L-Asparagine	−	+
Glycyl L-aspartic acid	−	+
Glycyl L-glutamic acid	−	+
Urocanic acid	−	+
Glycerol	−	+
Glucose 1-phosphate	−	+
Glucose 6-phosphate	−	+
DNA G+C content (mol%)	53.3	51–53

of respiratory quinones, cellular fatty acid composition and polar lipids. Polar lipids were identified by two-dimensional TLC (Minnikin *et al.*, 1984) and staining with molybdatophosphoric acid (Merck Millipore) for total lipids, molybdenum blue (Sigma-Aldrich) for phospholipids, anisaldehyde reagent for glycolipids or ninhydrin for aminolipids. The DNA G+C content was determined by the HPLC method of Tamaoka & Komagata (1984).

Strain MSd3^T contained only MK-7 as respiratory quinone. The major cellular fatty acids of strain MSd3^T were summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) (43.4 % of the total) and $C_{16:1}\omega5c$ (19.0 %) (percentages of other cellular fatty acids are shown in Table S1, available in the online Supplementary Material). The cellular fatty acid profile of strain MSd3^T was similar to that of *S. linguale* DSM 74^T (Table S1). Polar lipids of strain MSd3^T were phosphatidylethanolamine, three unidentified aminophospholipids and three unidentified polar lipids (Fig. S1). The DNA G+C content of strain MSd3^T was 53.3 mol%. These chemotaxonomic characteristics agreed with those of the genus *Spirosoma*.

DNA–DNA hybridization between strain MSd3^T and *S. linguale* DSM 74^T was conducted fluorometrically by the method of Ezaki *et al.* (1989). Photobiotin-labelled DNA was hybridized with unlabelled DNA which had been immobilized on the surface of microdilution wells. Hybridization was performed at 46 °C (T_m −45 °C). DNA–DNA relatedness between strain MSd3^T and *S. linguale* DSM 74^T was 19 % or 25 % (reciprocal value). These values of DNA–DNA relatedness were well below the 70 % cut-off point recommended for the assignment of bacterial strains to the same genomic species (Wayne *et al.*, 1987).

The phylogenetic position of strain MSd3^T indicated that this strain is a member of the genus *Spirosoma*. The phenotypic characteristics of strain MSd3^T agreed with those of this genus. Several differences in physiological characteristics and the DNA–DNA relatedness values between strains MSd3^T and *S. linguale* DSM 74^T indicated that strain MSd3^T should be classified in a species separate from *S. linguale*. We therefore propose that strain MSd3^T should be assigned to a novel species of the genus *Spirosoma*, for which the name *Spirosoma fluviale* sp. nov. is proposed.

Description of *Spirosoma fluviale* sp. nov.

Spirosoma fluviale (flu.vi.a′le. L. neut. adj. *fluviale* belonging to a river, the source of the type strain).

Cells are Gram-stain-negative, non-spore-forming, aerobic, non-motile, curved rods resulting in rings, coils and undulating filaments. After 3 days on R2A agar at 30 °C, colonies are chrome-yellow, circular, entire, convex, glistening and 3–4 mm in diameter. Grows at pH 6.0–8.0 (optimum, pH 7.0), at 4–30 °C (optimum, 25–30 °C), and with 0–1 % (w/v) NaCl (optimum, 0 %). Flexirubin-type pigments are absent. Catalase- and oxidase-positive. In API 20 NE tests, positive for β-glucosidase, β-galactosidase (PNPG), and assimilation of D-glucose, D-mannose, *N*-acetyl-D-glucosamine and maltose, but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis and assimilation of L- arabinose, D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. In API 20 E tests, negative for β-galactosidase (ONPG), arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophan deaminase, indole production, acetoin production, gelatinase and fermentation/oxidation of D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, melibiose, amygdalin and L-arabinose. In the Biolog GN2 system, the following carbon sources can be oxidized: glycogen, *N*-acetyl-D-glucosamine, D-arabitol, cellobiose, D-fructose, D-galactose, gentiobiose, α-D-glucose, α-lactose, lactulose, maltose, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, D-sorbitol, sucrose, trehalose, turanose, methyl pyruvate, monomethyl succinate, acetic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-ketoglutaric acid, DL-lactic acid, succinamic acid, L-alanine, L-alanylglycine, L-aspartic acid, L-glutamic acid, L-ornithine, uridine and 2,3-butanediol. In the Biolog GN2 system, the following carbon sources cannot be oxidized: α-cyclodextrin, dextrin, Tween 40, Tween 80, *N*-acetyl-D-galactosamine, adonitol, L-arabinose,

i-erythritol, L-fucose, *myo*-inositol, D-mannitol, L-rhamnose, xylitol, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, α-hydroxybutyric acid, *β*-hydroxybutyric acid, *γ*-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, L-alaninamide, D-alanine, L-asparagine, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, *γ*-aminobutyric acid, urocanic acid, inosine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. Resistant to polymyxin B. Sensitive to ampicillin, chloramphenicol, kanamycin, rifampicin, streptomycin and tetracycline. Respiratory quinone is MK-7. The major cellular fatty acids are summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) and $C_{16:1}\omega5c$. Polar lipids consist of phosphatidylethanolamine, three unidentified aminophospholipids and three unidentified polar lipids.

The type strain, MSd3^T (=JCM 30659^T=DSM 29961^T), was isolated from a freshwater sample collected from the Hosoda River in Wakamiya park (Atsugi, Kanagawa, Japan). The genomic DNA G+C content of the type strain is 53.3 mol% (HPLC method).

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