Spirosoma spitsbergense sp. nov. and *Spirosoma luteum* sp. nov., isolated from a high Arctic permafrost soil, and emended description of the genus *Spirosoma*

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Two pigmented, Gram-negative, non-motile, pleomorphic rod-shaped bacteria (strains SPM-9^T and SPM-10^T) were isolated from a permafrost soil collected from the Adventdalen valley, Spitsbergen, northern Norway. A third isolate (strain M5-H2) was recovered from the same soil sample after the sample had been exposed to simulated Martian environmental conditions. The three strains were characterized taxonomically by using a polyphasic approach. Phylogenetic, chemotaxonomic, physiological and morphological analyses demonstrated that the three isolates were most closely related to members of the genus *Spirosoma*. 16S rRNA gene sequence data indicated that the three isolates could be divided into two clusters: (i) strain SPM-9^T and (ii) strains SPM-10^T and M5-H2. This grouping was confirmed by DNA–DNA hybridization experiments. Strains SPM-9^T and SPM-10^T exhibited 92% 16S rRNA gene sequence similarity to both *Spirosoma linguale* LMG 10896^T and *Spirosoma rigui* WPCB 118^T. The major fatty acids present in all three isolates were summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1ω7c}; 43.0–48.2% of the total), C_{16:1ω5c} (19.1–21.3%), C_{16:0} (6.7–7.3%), iso-C_{17:0} 3-OH (4.7–6.0%) and iso-C_{15:0} (2.6–5.7%). On the basis of their phenotypic and genotypic characteristics, the new strains are assigned to two novel species of the genus *Spirosoma*, for which the names *Spirosoma spitsbergense* sp. nov. and *Spirosoma luteum* sp. nov. are proposed. The type strain of *Spirosoma spitsbergense* is SPM-9^T (=NCIMB 14407^T=DSM 19989^T) and the type strain of *Spirosoma luteum* is SPM-10^T (=NCIMB 14406^T=DSM 19990^T). An emended description of the genus *Spirosoma* is also proposed.

The family 'Flexibacteraceae' is one of the major phylogenetic lineages within the phylum *Bacteroidetes* (Garrity & Holt, 2001). A polyphasic taxonomic approach (Vandamme *et al.*, 1996) driven by 16S rRNA gene sequenced-based phylogeny has expanded the discovery and description of members of this family. Phylogenetically, the group is diverse and includes the genera *Arcicella*, *Cyclobacterium*, *Spirosoma*, *Larkinella*, *Dyadobacter*, *Flectobacillus*, *Flexibacter*, *Microscilla* and *Runella*. At the time of writing, the genus *Spirosoma* comprises two recognized species, *Spirosoma linguale* (Larkin & Borrall, 1984) and *Spirosoma rigui* (Baik *et al.*, 2007). Cells of these species are Gram-negative, non-

spore-forming, yellow-pigmented rods that exhibit gliding motility (Baik *et al.*, 2007; O. Nedashkovskaya, personal communication). Characteristic long filaments and coils may also be produced.

During the course of a study investigating the biodiversity of permafrost soil collected from Spitsbergen in the high Arctic, two bacterial strains displaying the general characteristics of members of the genus *Spirosoma* were isolated (Hansen *et al.*, 2007). Strain SPM-9^T produced a yellow diffusible pigment while strain SPM-10^T produced a dark orange non-diffusible pigment. A third isolate, M5-H2, was recovered from the same permafrost soil sample after the sample had been exposed to simulated Martian conditions in a Mars simulation chamber (Jensen *et al.*, 2008). Strain M5-H2 produced a deep orange non-diffusible pigment and appeared to be closely related to isolate SPM-10^T.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains SPM-9^T and SPM-10^T are EF451725 and EF451726, respectively.

When cultured in R2A broth (Difco), the cells formed long filaments and distinctive coils. Preliminary 16S rRNA gene sequencing indicated that strains SPM-9^T and SPM-10^T were most closely related, albeit at a relatively low level of similarity, to *S. linguale* LMG 10896^T and *S. rigui* WPCB 118^T. These data suggested that strains SPM-9^T, SPM-10^T and M5-H2 may represent novel species of the genus *Spirosoma*. The novel strains were therefore subjected to a detailed taxonomic investigation.

Strains SPM-9^T and SPM-10^T were isolated from a permafrost soil sample collected in the Adventdalen valley, Spitsbergen (78° 12' N 15° 50' E), northern Norway, by using a standard dilution plating technique. Isolation was achieved on R2A agar (Difco) at 15 °C. The isolates were routinely cultured on R2A agar except when large-scale production of cells was required for chemotaxonomic analyses, for which R2A broth was used.

Bacterial DNA preparation and PCR amplification of the 16S rRNA gene were performed as described by Hansen *et al.* (2007). The resultant 16S rRNA gene sequences (1182 bp) were aligned and compiled by using the ARB program package (Ludwig *et al.*, 2004) and were added to the alignment of the Silva SSURef version 92 ARB database (Pruesse *et al.*, 2007). From this database, a range of sequences affiliated with the order 'Sphingobacteriales' was selected for phylogenetic analyses. A phylogenetic tree was inferred by neighbour-joining analysis (with the Jukes–Cantor distance correction). A 50 % conservation filter (Ludwig *et al.*, 1998) was applied for selecting sequence positions (1182) for the analysis. The filter was calculated for *Escherichia coli* positions 103–1340, which were spanned by all sequences. Bootstrap analyses (100 replications) based on the distance matrix (with the Jukes–Cantor distance correction) and maximum-parsimony methods were performed by using PAUP* version 4.0b10 (Swofford, 2003) and the ARB program package (Ludwig *et al.*, 2004), respectively. All unambiguously aligned sequence positions (1227) between *E. coli* positions 103 and 1340 were included in the bootstrap analyses. Fig. 1 shows that the three new isolates formed a distinct cluster together with their nearest phylogenetic neighbours, namely *S. rigui* WPCB 118^T and *S. linguale* LMG 10896^T. Strains SPM-9^T and SPM-10^T showed 92 % 16S rRNA gene similarity to *Spirosoma rigui* WPCB 118^T. Strains SPM-9^T and SPM-10^T shared 96 % 16S rRNA gene sequence similarity. Strains SPM-10^T and M5-H2 shared 98 % 16S rRNA gene sequence similarity, suggesting that they may belong to the same species.

The temperature range for growth of the three new isolates was tested on R2A plates incubated at 4–40 °C in increments of approximately 5 °C. The pH range for growth (pH 5–9 in 1 pH unit increments) was determined in R2A broth by assessing changes in OD₆₀₀ of the cultures incubated at 25 °C on an orbital shaker at 100 r.p.m. for 10 days as compared with an uninoculated control. The pH of the medium was adjusted by the addition of either

1 M HCl or 1 M NaOH and was checked after autoclaving. Tolerance to NaCl was determined by amending R2A agar with NaCl to reach final concentrations of 0, 1, 2, 3, 4 and 5 % NaCl (w/v).

Gram staining was carried out according to the method described by Barrow & Feltham (1993). Cell morphology, the presence of gas vacuoles and motility were examined in wet mount preparations by phase-contrast microscopy with an Olympus BH2 microscope. The absence of gas vacuoles was confirmed by exposing the cells to a sudden change in pressure and observing whether they sank to the bottom of the growth medium. Gliding motility was investigated according to the method of Bowman (2000), except that one-quarter strength R2A broth was used instead of one-quarter strength marine broth 2216 (Difco). The ability of the isolates to grow anaerobically was determined on R2A agar by using the Oxoid Anaerobic System (Oxoid Ltd). Pigment absorption spectra between 300 and 750 nm were taken by using a Pye-Unicam Helios lambda spectrophotometer after methanol extraction (Gosink *et al.*, 1998). The presence of flexirubin pigments was investigated by using the KOH test as described by Bernardet *et al.* (2002). Biochemical tests, including nitrate reduction, urease, oxidase and catalase activity and indole production, were performed according to Smibert & Krieg (1994). Additional biochemical tests were performed by using API 20NE strips (bioMérieux) and GN2 plates (Biolog Inc.) according to the manufacturers' instructions. Sensitivity to antibiotics was determined by using the disc-diffusion method of Bauer *et al.* (1966) on R2A agar plates incubated at 25 °C and inspected after 3 days. Gelatin hydrolysis and amylase and alkaline phosphatase activities were determined according to the methods described by Barrow & Feltham (1993). Detailed results of the morphological, physiological and biochemical analyses are given in Table 1 and in the species descriptions below.

As shown in Table 1, a number of phenotypic characteristics could be used to distinguish strains SPM-9^T, SPM-10^T and M5-H2 from their closest phylogenetic neighbour (*S. rigui* WPCB 118^T), for example the absence of gliding motility, growth temperature maximum, presence of alkaline phosphatase activity, absence of β -galactosidase activity, sensitivity to the antibiotics kanamycin and gentamicin, and inability to grow under anoxic conditions. Similarly, the three new isolates could be differentiated from *S. linguale* LMG 10896^T on the basis of growth temperature maximum, absence of ring-like and horse-shoe-shaped cells, sensitivity to penicillin G and neomycin, and inability to utilize fructose, maltose, xylose, starch or cellobiose.

The cellular fatty acid composition of the three isolates was determined according to the protocols of the Microbial Identification System (MIDI). Cultures were grown in R2A broth at 25 °C on an orbital shaker at 100 r.p.m. for 5 days. The major fatty acids present in all three isolates were summed feature 3 (comprising iso-C_{15:0} 2-OH and/or

Fig. 1. Phylogenetic tree constructed from a neighbour-joining analysis of 16S rRNA gene sequences showing the inferred phylogenetic position of strains M5-H2, SPM-9^T and SPM-10^T among selected taxa affiliated with the family 'Flexibacteraceae'. Distance-matrix- and maximum-parsimony-based bootstrap percentage values (>50 %) are indicated above and below nodes, respectively. Bar, 5 % estimated sequence divergence. The following taxa (not shown) were used as outgroups: *Adhaeribacter aquaticus* DSM 16391^T (AJ626894), [*Flexibacter*] *aggregans* ATCC 23162^T (M64628), [*Flexibacter*] *litoralis* DSM 6794^T (AB078056), [*Flexibacter*] *polymorphus* DSM 9678^T (AB078059), *Roseivirga seohaensis* JCM 12600^T (AY739663), *Perexilibacter aurantiacus* JCM 23194^T (AB276355), *Pontibacter actinarius* LMG 23027^T (AY989908), *Rapidithrix thailandica* JCM 23209^T (AB284517) and *Thermonema rossianum* DSM 10300^T (Y08956).

C_{16:1}ω7c; 43.0–48.2 % of the total), C_{16:1}ω5c (19.1–21.3 %), C_{16:0} (6.7–7.3 %), iso-C_{17:0} 3-OH (4.7–6.0 %) and iso-C_{15:0} (2.6–5.7 %). As shown in Table 2, the cellular fatty acid profiles of isolates SPM-9^T, SPM-10^T and M5-H2 were similar to those reported by Vancanneyt *et al.* (2006) and Baik *et al.* (2007) for *S. linguale* LMG 10896^T and *S. rigui* WPCB 118^T, respectively. However, the growth conditions used for fatty acid analysis of these two strains were not specified. Analysis of respiratory quinones was performed by the Identification Service of the Deutsche Sammlung von Micro-organismen und Zellkulturen (DSMZ) and Dr Brian Tindall, Braunschweig, Germany. All three isolates contained MK-7 as the predominant menaquinone, in accordance with the results for *S. linguale* (Urakami & Komagata, 1986) and *S. rigui* (Baik *et al.*, 2007).

DNA–DNA hybridization experiments were also performed by the Identification Service of the DSMZ by using the method of De Ley *et al.* (1970) as modified by Huß *et al.* (1983). Strains SPM-10^T and M5-H2 showed a level of DNA–DNA relatedness of 98.4 %, demonstrating that they belong to the same species. In contrast, strains SPM-9^T and SPM-10^T showed a level of DNA–DNA relatedness of only 17.4 %, confirming that they belong to separate species. The DNA G + C contents of strains SPM-9^T, SPM-10^T and M5-H2 as determined by reversed-phase HPLC (Mesbah *et al.*, 1989) were 49.1, 50.2 and 51.0 mol%, respectively.

On the basis of 16S rRNA gene sequence data and the results of phenotypic and phylogenetic analyses, strain SPM-9^T and strains SPM-10^T and M5-H2 are considered

to represent two novel species of the genus *Spirosoma*, for which the names *Spirosoma spitsbergense* sp. nov. and *Spirosoma luteum* sp. nov., respectively, are proposed. The phenotypic characteristics that differentiate these two novel species are detailed in Table 1. On the basis of the data presented in this study together with that reported by Vancanneyt *et al.* (2006) and Baik *et al.* (2007), an emended description of the genus *Spirosoma* is also proposed.

Emended description of the genus *Spirosoma* Migula 1894

The description is as given by Larkin & Borrall (1984) with the following modifications. Cells are non-motile or motile by means of gliding. Yellow or orange, diffusible or non-diffusible pigments that do not belong to the flexirubin type are produced. Oxidase activity is species-dependent. Strictly aerobic or facultatively anaerobic. The predominant respiratory quinone is menaquinone 7 (MK-7) and the major cellular fatty acids are summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), C_{16:1}ω5c, iso-C_{15:0} and C_{16:0}. The DNA G + C content is 49–53 mol%.

Description of *Spirosoma spitsbergense* sp. nov.

Spirosoma spitsbergense (spits.berg.en'se. N.L. neut. adj. *spitsbergense* pertaining to Spitsbergen in the high Arctic, northern Norway, from where the type strain was isolated).

Table 1. Phenotypic properties that distinguish strains SPM-9^T, SPM-10^T and M5-H2 from their closest phylogenetic neighbours

Strains: 1, SPM-9^T; 2, SPM-10^T; 3, M5-H2; 4, *S. linguale* LMG 10896^T; 5, *S. rigui* WPCB 118^T. Data for *S. linguale* LMG 10896^T and *S. rigui* WPCB 118^T are from Larkin & Borrall (1984), Vancanneyt *et al.* (2006), Baik *et al.* (2007) and O. Nedashkovskaya (personal communication). All strains are catalase-positive, utilize glucose and are unable to utilize arabinose, mannitol or citrate. All strains (with the exception of *S. linguale* LMG 10896^T, which was not tested) are unable to utilize gluconate, caprate, adipate or glycerol. All strains are sensitive to ampicillin, bacitracin and tetracycline and grow optimally in the absence of NaCl. None of the strains is able to reduce nitrate, and gas vacuoles are not produced. +, Positive; –, negative; w, weakly positive; ND, not determined; A, aerobic; FA, facultatively anaerobic; R, resistant; S, susceptible.

Characteristic	1	2	3	4	5
Cell morphology	Straight rods	Straight rods	Straight rods	Ring-shaped	Straight rods
Cell size (length × width; µm)	4–10 × 1	4–10 × 1	4–8 × 1.2	6 × 1	1.5–10.8 × 1.0
Gliding motility	–	–	–	+	+
Reaction to oxygen	A	A	A	A	FA
Pigmentation	Yellow	Orange	Orange	Yellow	Yellow
Diffusible pigment	+	–	–	–	–
Growth temperature range (°C)	4–30	4–30	4–30	5–39	4–37
Antibiotic sensitivity					
Chloramphenicol	S	R	R	R	S
Gentamicin	S	S	S	S	R
Kanamycin	S	S	S	S	R
Nalidixic acid	R	R	R	ND	S
Neomycin	S	S	S	R	ND
Penicillin G	S	S	S	R	S
Vancomycin	R	R	R	ND	S
Enzymic activities					
Aesculin hydrolysis	–	+	+	ND	+
Alkaline phosphatase	+	+	+	+	–
Amylase	–	–	–	+	ND
Arginine dihydrolase	–	–	–	+	ND
β-Galactosidase	–	–	–	+	+
Oxidase	w	–	–	+	–
Urease	+	–	–	–	ND
Carbon source utilization					
Cellobiose	–	–	–	+	–
Fructose	–	–	–	+	–
Malate	–	+	+	ND	–
Maltose	–	–	–	+	–
Mannose	+	–	–	+	–
Pyruvate	–	+	+	+	ND
Starch	–	–	–	+	–
D-Xylose	–	–	–	+	–
DNA G + C content (mol%)	49.1	50.2	51.0	51–53	53.3

Cells are Gram-negative rods, 1 µm in diameter and 4–10 µm in length, non-motile, non-spore-forming and devoid of gas vacuoles. A diffusible yellow pigment that does not belong to the flexirubin type is produced. When grown in R2A broth, cells form long filaments up to 80 µm long. Colonies on R2A agar are circular with a diameter of 0.5–1.5 mm after 2 weeks incubation at 25 °C. Growth occurs aerobically at 4–30 °C (optimum, 25 °C) and at pH 6–9 (optimum, pH 7). Cells grow optimally in the absence of NaCl but are able to tolerate 1 % NaCl. Positive catalase reaction and weakly positive oxidase reaction. No growth is observed under anaerobic conditions. Nitrate is not reduced. H₂S and indole are not produced. Arginine is not hydrolysed. Positive for urease activity but negative for

amylase, β-galactosidase and lipase activities and for aesculin hydrolysis. Glucose and mannose are utilized as carbon and energy sources. The following substrates are not utilized: arabinose, fructose, mannitol, maltose, gluconate, caprate, adipate, malate, citrate, pyruvate, xylose, starch, glycerol and cellobiose. Susceptible to the following antibiotics (µg per disc): ampicillin (33), penicillin G (10), chloramphenicol (60), gentamicin (40), streptomycin (20), neomycin (20), kanamycin (30) and tetracycline (50); resistant to (µg per disc): bacitracin (60), vancomycin (70) and nalidixic acid (30). The major cellular fatty acids are summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c), C_{16:1}ω5c, iso-C_{15:0} and C_{16:0}; the complete fatty acid profile is given in Table 2. The predominant menaquinone is MK-7.

Table 2. Cellular fatty acid profiles of strains SPM-9^T, SPM-10^T and M5-H2 and their closest phylogenetic neighbours

Strains: 1, SPM-9^T; 2, SPM-10^T; 3, M5-H2; 4, *S. linguale* LMG 10896^T; 5, *S. rigui* WPCB 118^T. Data for *S. linguale* LMG 10896^T and *S. rigui* WPCB 118^T are from Vancanneyt *et al.* (2006) and Baik *et al.* (2007). Fatty acids amounting to less than 1 % of the total fatty acids in all the strains listed are not shown. tr, Trace (<1 %); –, not detected.

Fatty acid	1	2	3	4	5
Straight chain saturated					
C _{14:0}	0.5	0.3	0.3	–	1.3
C _{15:0}	0.3	0.3	–	–	–
C _{16:0}	7.0	7.3	6.8	–	8.8
C _{18:0}	–	–	–	–	1.2
C _{16:0} 3-OH	4.5	3.5	3.4	–	2.8
Branched saturated					
iso-C _{13:0}	–	–	–	3.8	–
iso-C _{15:0}	5.7	2.6	3.1	7.6	9.5
iso-C _{15:0} 3-OH	2.3	2.5	2.7	4.9	2.6
iso-C _{16:0} 3-OH	0.7	–	–	–	–
iso-C _{17:0} 3-OH	6.1	4.7	6.1	6.5	3.5
anteiso-C _{15:0} 3-OH	1.1	0.9	0.8	tr	1.3
Mono-unsaturated					
C _{16:1} ω5c	21.3	19.1	20.4	17.5	18.5
C _{18:1} ω7c	–	–	–	–	1.5
Summed feature 3*	42.9	45.0	48.2	47.9	45.6

*Summed features represent fatty acids that could not be separated by GLC with the MIDI system; summed feature 3 comprises iso-C_{15:0} 2-OH and/or C_{16:1}ω7c.

The type strain, SPM-9^T (=NCIMB 14407^T=DSM 19989^T), was isolated from a permafrost soil sample in the Adventdalen valley, Spitsbergen, northern Norway. The DNA G+C content of the type strain is 49.1 mol%.

Description of *Spirosoma luteum* sp. nov.

Spirosoma luteum (lu'te.um. L. neut. adj. *luteum* orange coloured).

Cells are Gram-negative rods, 1 µm in diameter and 4–10 µm in length, non-motile, non-spore-forming and devoid of vacuoles. A non-diffusible orange pigment that does not belong to the flexirubin type is produced. When grown in R2A broth, cells form long filaments up to 80 µm long. Colonies on R2A agar are circular with a diameter of 1–2 mm after 2 weeks incubation at 25 °C. Growth occurs aerobically at 4–30 °C (optimum, 25 °C) and at pH 6–9 (optimum, pH 7). Cells grow optimally in the absence of NaCl but are able to tolerate 1 % NaCl. Positive catalase reaction and negative oxidase reaction. No growth is observed under anaerobic conditions. Nitrate is not reduced. H₂S and indole are not produced and arginine is not hydrolysed. Negative for urease, amylase, β-galactosidase and lipase activities. Glucose, malate,

pyruvate and aesculin are utilized as carbon and energy sources. The following substrates are not utilized: arabinose, fructose, mannitol, mannose, maltose, gluconate, caprate, adipate, citrate, xylose, starch, glycerol and cellobiose. Susceptible to the following antibiotics (µg per disc): ampicillin (33), penicillin G (10), gentamicin (40), streptomycin (20), neomycin (20), kanamycin (30) and tetracycline (50); resistant to (µg per disc): bacitracin (60), vancomycin (70), nalidixic acid (30) and chloramphenicol (60). The major cellular fatty acids are summed feature 3 (comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c, C_{16:1}ω5c, iso-C_{15:0} and C_{16:0}); the complete fatty acid profile is given in Table 2. The predominant menaquinone is MK-7.

The type strain, SPM-10^T (=NCIMB 14406^T=DSM 19990^T), was isolated from a permafrost soil sample in the Adventdalen valley, Spitsbergen, northern Norway. The DNA G+C content of the type strain is 50.2 mol%. M5-H2 is a second strain of the species.

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