

## *Spongiispira norvegica* gen. nov., sp. nov., a marine bacterium isolated from the boreal sponge *Isops phlegraei*

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The bacterial strain Gp\_4\_7.1<sup>T</sup>, isolated from the marine sponge *Isops phlegraei* collected at the Sula Ridge off the Norwegian coast, was characterized. The isolate was a motile spirillum that was monopolarly and monotrichously flagellated. It was aerobic, Gram-negative, oxidase-positive and catalase-negative. Optimal growth occurred between 20 and 30 °C, at pH 7–8 and with a salt concentration of 2–3 % (w/v). The isolate showed a relatively restricted nutritional profile. Substrate utilization tests were only positive for arabinose. Enzyme tests were positive for esterase lipase C8, lipase C14, leucine arylamidase and naphthol-AS-BI-phosphohydrolase. The strain was not able to reduce nitrate. The major cellular fatty acids were C<sub>16:1ω7</sub> and C<sub>16:0</sub>. The DNA G+C content was 62.6 mol%. Phylogenetic analysis based on 16S rRNA gene sequence comparison classified the strain as a member of the order *Oceanospirillales* in the class *Gammaproteobacteria*. Strain Gp\_4\_7.1<sup>T</sup> formed a distinct phyletic line with less than 94 % 16S rRNA gene sequence similarity to its closest relatives with validly published names. Based on the determined data, it is proposed that the strain represents a novel species in a new genus, *Spongiispira norvegica* gen. nov., sp. nov.; the type strain of *Spongiispira norvegica* is Gp\_4\_7.1<sup>T</sup> (=DSM 17749<sup>T</sup> =NCIMB 14401<sup>T</sup>).

In recent years, investigations of marine sponges have been of great interest and have focussed on analysis of sponge-associated bacteria, as well as on the search for compounds with pharmacological benefit (Taylor *et al.*, 2007). Up to now, most studies concerning sponge-associated bacteria have concentrated on porifera from tropical, subtropical

and temperate regions. In contrast, investigations of the microbial assemblage from cold-water sponges are rare (Althoff *et al.*, 1998; Thiel *et al.*, 2002; Webster *et al.*, 2004; Pape *et al.*, 2006; Hoffmann *et al.*, 2005, 2006). The diversity of sponge-associated micro-organisms has been investigated by different methods, such as cloning of the 16S rRNA genes (Webster *et al.*, 2001; Hentschel *et al.*, 2002; Webster *et al.*, 2004; Fieseler *et al.*, 2004; Li & Liu, 2006; Thiel *et al.*, 2007), denaturing gradient gel electrophoresis analysis (Thoms *et al.*, 2003; Taylor *et al.*, 2004;

Abbreviation: FAMES, fatty acid methyl esters.

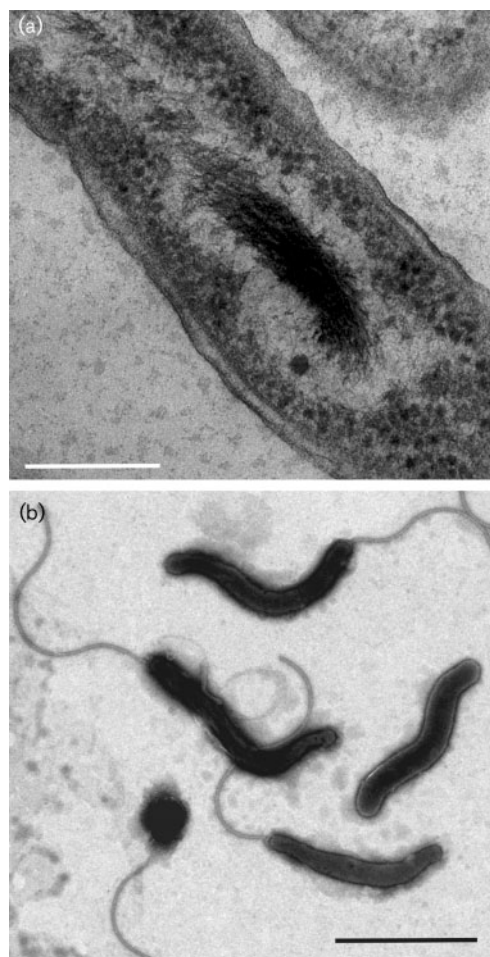
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Spongiispira norvegica* strain Gp\_4\_7.1<sup>T</sup> is AM117931.

Li *et al.*, 2006; Wichels *et al.*, 2006), fluorescence *in situ* hybridization (Webster *et al.*, 2001; Pape *et al.*, 2006; Hoffmann *et al.*, 2006), metagenomic analysis (Piel *et al.*, 2005; Schirmer *et al.*, 2005; Fieseler *et al.*, 2006) and different cultivation approaches (Dieckmann *et al.*, 2005; Lafi *et al.*, 2005; Olson & McCarthy, 2005; Sfanos *et al.*, 2005). Among the different phylogenetic bacterial groups isolated from sponges, bacteria with an affiliation to the class *Gammaproteobacteria*, particularly with the genera *Alteromonas*, *Pseudomonas*, *Pseudoalteromonas* and *Vibrio* (Hentschel *et al.*, 2001; Dieckmann *et al.*, 2005; Sfanos *et al.*, 2005), have been reported frequently. To our knowledge, sponge-associated bacteria affiliated to the order *Oceanospirillales* within the *Gammaproteobacteria* have only been reported in a few publications (Sfanos *et al.*, 2005; Li & Liu, 2006). These strains were detected from different demosponges collected from various subtropical and tropical locations and were not related to isolate Gp\_4\_7.1<sup>T</sup>, which is described in this study.

Strain Gp\_4\_7.1<sup>T</sup> was isolated from the cold-water sponge *Isops phlegraei* (class Demospongiae) collected from the Sula Ridge off the coast of mid-Norway and could only be isolated from this one sponge out of 11 demosponges investigated during a sampling campaign in August 1999. The sponge was collected from a depth of approximately 320 m. Sections of the sponge were homogenized, serially diluted in filter-sterilized seawater and plated on marine broth agar (MBA; Difco). Cultivation was performed at 8 °C. Colony morphology was analysed by stereomicroscopy from colonies grown on MBA plates after 5 days. The isolate formed beige, circular colonies with a diffuse and flat shape. The colonies grew into the agar layer and, for transfer, they had to be lifted out together with the surrounding medium. The colony size after 5 days on MBA at room temperature was 0.2–0.6 mm. Colony morphology showed no analogy with that of *Oleispira antarctica* (Yakimov *et al.*, 2003), phylogenetically the most closely related species with a validly published name.

Cell morphology was examined by transmission electron microscopy using a Philips CM100 electron microscope. Cells were grown for 5 days in sterile-filtered marine broth (MB; Difco), concentrated by centrifugation and negatively stained by uranyl acetate according to Steven *et al.* (1988). Cells were embedded in epoxy resin according to Spurr (1969) for examination of ultrathin sections. Motility was examined by phase-contrast microscopy of bacterial cells grown in MB for 2 days at room temperature. Cells were Gram-negative (Fig. 1a), non-spore-forming, monopolarly and monotrichously flagellated, motile spirilla (2.0–5.0 × 0.3–0.4 µm) (Fig. 1b). The cell morphologies of Gp\_4\_7.1<sup>T</sup> and *O. antarctica* were very similar; however, the particular morphological feature of the drumstick-like thickening of the ends of the cell, described for *O. antarctica*, could not be observed for Gp\_4\_7.1<sup>T</sup>.

Temperature range and optimum for growth were tested at 2–40 °C in MB. The range of pH and optimum for growth



**Fig. 1.** Transmission electron micrographs of cells of strain Gp\_4\_7.1<sup>T</sup>. (a) Cell shape and nuclear material are shown after ultrathin sectioning. Bar, 0.2 µm. (b) Monopolarly flagellated spirilla are shown after negative staining with 0.8% uranyl acetate. Bar, 2 µm.

were determined from pH 5 to 10 at 30 °C in a medium containing 1 g peptone l<sup>-1</sup>, 5 g yeast extract l<sup>-1</sup> and 3% (w/v) artificial seawater (Lyman & Fleming, 1940). NaCl concentration range and optimum for growth were analysed at 30 °C for 0–3% (w/v) in medium consisting of 1 g peptone l<sup>-1</sup>, 5 g yeast extract l<sup>-1</sup> and 0–3% (w/v) artificial seawater. Growth tests with salt concentrations of 4–9% (w/v) were done in medium containing 1 g peptone l<sup>-1</sup>, 5 g yeast extract l<sup>-1</sup> and 3% (w/v) artificial seawater, with the addition of 1–6% (w/v) NaCl. Anaerobic growth was tested on MBA in glass tubes under anaerobic conditions (80% N<sub>2</sub>/20% CO<sub>2</sub>) and by using the Anaerocult System (Merck). Catalase and oxidase activities were tested with kits (Merck) according to the manufacturer's manual. Isolate Gp\_4\_7.1<sup>T</sup> was aerobic, oxidase-positive, catalase-negative and unable to reduce nitrate. It grew at pH 6.5–8.5 and 6–37 °C, with optimum growth at pH 7–8 and 20–30 °C. Growth was observed in media with

salt concentrations of 2–7% (w/v), with optimum growth in 2–3% (w/v) artificial seawater. Growth was not detected with NaCl as sole salt source. In comparison, *O. antarctica* is able to reduce nitrate, is catalase-positive, grows at –6 to +28 °C (optimum at 2–4 °C) and grows in NaCl concentrations of 1–10% (w/v) [optimum at 3–5% (w/v)]. *O. antarctica* can grow well with NaCl as sole salt source, in contrast to Gp\_4\_7.1<sup>T</sup>.

Physiological characterization was performed with microplates for Gram-negative bacteria (Biolog), API ZYM and API 20E (both bioMérieux). After 4 days of growth in MB at 18 °C, bacteria were resuspended in artificial seawater for Biolog and in 0.6% (w/v) NaCl for API ZYM and API 20E. The test strips/wells were inoculated as described in the manufacturers' manuals and incubated at 18 °C. API ZYM and API 20E were read after 2 days; Biolog was kept for up to 5 days. All physiological tests were performed in duplicate. Gp\_4\_7.1<sup>T</sup> showed high esterase lipase C8 and lipase C14 activities, as well as weak leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities. Furthermore, the isolate was able to utilize arabinose for growth. Additional tested substrates, e.g. different carbohydrates and amino acids, could not be used, which suggests a relatively restricted nutritional profile. Gp\_4\_7.1<sup>T</sup> and *O. antarctica* both show lipase activity and cannot metabolize most of the tested carbohydrates and amino acids. The phenotypic and chemotaxonomic characteristics that differentiate the novel isolate Gp\_4\_7.1<sup>T</sup> from *O. antarctica* are summarized in Table 1. Isolate Gp\_4\_7.1<sup>T</sup> is also related phylogenetically to various *Pseudomonas* species, but with lower similarities than to *O. antarctica*. Phenotypic and chemotaxonomic characteristics are so different from those of *Pseudomonas* species that a closer relationship can be ruled out in these cases, i.e. the cell morphology of *Pseudomonas* species is always straight or slightly curved rods, they can utilize a wide variety of organic compounds and they are always catalase-positive.

Whole-cell fatty acid methyl esters (FAMES) were obtained from washed cells by extraction and transesterification of 20 mg freeze-dried cells with a mixture of trimethylchlorosilane/methanol (1:8, v:v; 1 h, 75 °C) and re-extraction with n-hexane. Compounds were analysed by GC and combined GC-MS. Quantification of individual compounds was achieved by adding heneicosanoic acid methyl ester (C<sub>21:0</sub>) as an internal standard of known concentration prior to GC analysis. FAMES were identified by comparison of mass spectra and retention times with published data and/or reference compounds. Double-bond positions of mono-unsaturated FAMES were determined from their dimethyl disulfide derivatives (Buser *et al.*, 1983). Hydroxy fatty acids were treated with bis(trimethylsilyl)trifluoroacetamide and analysed as their *o*-trimethylsilyl derivatives. For structural elucidations, monounsaturated FAMES were converted to their saturated structural analogues by hydrogenation. Double-bond positions are indicated from the methyl ( $\omega$ ) end of the fatty acid. Sixteen fatty acids containing 10–18 carbon atoms were found upon transesterification of cell

**Table 1.** Phenotypic and physiological characteristics that differentiate strain Gp\_4\_7.1<sup>T</sup> from the related species *Oleispira antarctica*

Data for *O. antarctica* were taken from Yakimov *et al.* (2003). Both organisms are monopolarly and monotrichously flagellated, motile, oxidase-positive spirilla and show lipase activity. Leucine arylamidase and naphthol-AS-BI-phosphohydrolase activity are detected in strain Gp\_4\_7.1<sup>T</sup> but no data are available for *O. antarctica*. Utilization of arabinose is weakly positive in strain Gp\_4\_7.1<sup>T</sup> but no data are available for *O. antarctica*. Utilization of hexadecane was detected in *O. antarctica* but no data are available for strain Gp\_4\_7.1<sup>T</sup>. ND, No data available; +, positive; –, negative; w, weak.

Characteristic	Gp_4_7.1 <sup>T</sup>	<i>O. antarctica</i>
Cell size ( $\mu$ m)		
Length	2–5	2–5
Diameter	0.3–0.4	0.4–0.8
Growth temperature (°C)		
Range	6–37	–6 to +28
Optimum	20–30	2–4
Growth pH		
Range	6.5–8.5	ND
Optimum	7–8	ND
Salt tolerance for growth (%)		
Range	2–7	1–10
Optimum	2–3	3–5
Catalase activity	–	+
Nitrate reduction to nitrite	–	+
DNA G+C content (mol%)	62.6	41–42

material of Gp\_4\_7.1<sup>T</sup>. The most abundant fatty acids by far were C<sub>16:1</sub> $\omega$ 7 (39.4%), C<sub>16:0</sub> (29.7%), C<sub>18:0</sub> (8.1%), C<sub>18:1</sub> $\omega$ 9 (6.9%), C<sub>10:0</sub> 3-OH (6.2%) and C<sub>18:1</sub> $\omega$ 7 (3.6%). In addition, several fatty acids were found in low concentrations: C<sub>16:0</sub> 3-OH (0.3%), C<sub>12:1</sub> $\omega$ 7 (0.8%), C<sub>14:1</sub> $\omega$ 9 (0.1%), C<sub>14:1</sub> $\omega$ 7 (0.2%), C<sub>16:1</sub> $\omega$ 9 (1.0%), C<sub>12:0</sub> (0.2%), C<sub>14:0</sub> (1.3%), C<sub>15:0</sub> (0.3%), C<sub>17:0</sub> (1.3%) and iso-C<sub>18:0</sub> (0.3%). Of the fatty acids identified, 41.2% were saturated and 52.0% were unsaturated. Differences in the cellular fatty acid compositions of Gp\_4\_7.1<sup>T</sup> and closely related *O. antarctica* were small.

The DNA G+C content was analysed at the DSMZ, Braunschweig, Germany. Calibration of the method was performed using non-methylated lambda-DNA (Sigma) possessing a known G+C content of 49.9 mol% (Mesbah *et al.*, 1989), as well as three genomic DNAs for which complete genome sequences have been published [*Bacillus subtilis* DSM 402 (G+C content of 43.5 mol%), *Xanthomonas campestris* pv. *campestris* DSM 3586<sup>T</sup> (G+C content of 65.1 mol%) and *Streptomyces violaceoruber* DSM 40783 (G+C content of 72.1 mol%)]. The DNA G+C content of Gp\_4\_7.1<sup>T</sup> was 62.6 mol%. This G+C content differs from that of *O. antarctica* (41–42 mol%).

For the phylogenetic characterization, DNA was extracted from bacterial cultures growing in MB medium, washed

with PBS and incubated at 95 °C for 15 min with alkaline lysis buffer [0.25 % (w/v) SDS, 50 mM NaOH]. Amplification of the nearly full-length 16S rRNA gene (1452 bp) was performed with the bacterial primers 616V (5'-AGAGTTTGATYMTGGCTCAG; *Escherichia coli* positions 8–27), 1525R (5'-AAGGAGGTGWTCARCC; 1525–1541), 610R (5'-ACCGCGGCTGCTGGCAC; 515–531), nonEUB (5'-ACTCCTACGGGAGGCAGC; 338–355), 699R (5'-RGGGTTGCGCTCGTT; 1101–1115), 699V (5'-AACGAGCGCAACCCY; 1101–1115), 609V (5'-GGM-TTAGATACCCBRGTAGT; 785–804) and 609R2 (5'-ACTACYVGGGTATCTAAKCC; 785–804). Sequence data were analysed with an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Phylogenetic affiliation was analysed with the ARB software (Ludwig *et al.*, 2004) and data were compared to the EMBL/GenBank nucleotide sequence database using BLAST. The phylogenetic tree was calculated with partial 16S rRNA gene sequences (1403 bp; *E. coli* position 56–1459) by using the neighbour-joining (Jukes–Cantor correction), maximum-parsimony and maximum-likelihood methods implemented in ARB. Different filters were used for the calculations. Comparisons with 16S rRNA gene sequences available in EMBL/GenBank and analysis by the ARB software indicated that Gp\_4\_7.1<sup>T</sup> belongs to the class *Gammaproteobacteria*. Within this class, the strain was most closely related to a number of marine bacteria that included members of the genus *Oleispira*. These strains included an uncultured bacterium (GenBank accession no. AY697896; 96 % sequence similarity; Prabakaran *et al.*, 2007) analysed from subantarctic seawater, a bacterium (DQ521390; 93 % sequence similarity) detected in Arctic and Antarctic sea ice and *O. antarctica* strains (AJ426420, AJ426421; 93 % sequence similarity; Yakimov *et al.*, 2003) from Antarctic coastal seawater. *O. antarctica* is the nearest neighbour phylogenetically with a validly published name. Furthermore, diverse uncultured bacteria from Arctic sea ice (GenBank accession no. AF468291, AF468308; Brinkmeyer *et al.*, 2003), Antarctic sea ice (DQ906763)

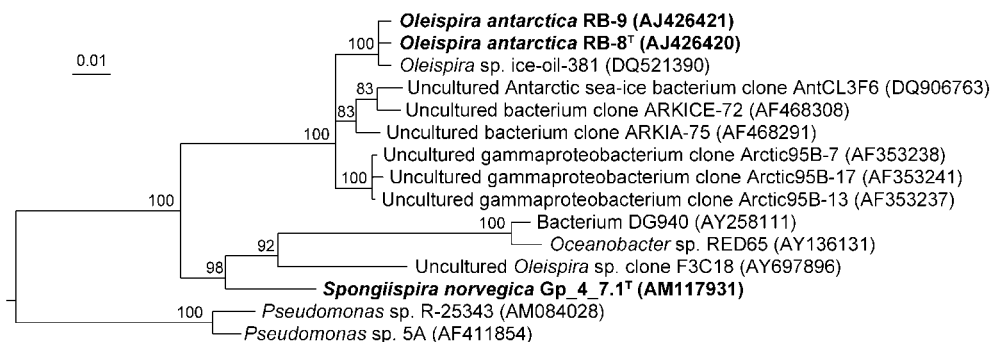
and from bacterioplankton of the Arctic Ocean (AF353238, AF353241, AF353237; Bano & Hollibaugh, 2002) showed 93–94 % sequence similarity to strain Gp\_4\_7.1<sup>T</sup>. It is noticeable that all bacteria mentioned above are from Arctic and Antarctic marine regions. Only strain DG940 (GenBank accession no. AY258111; Green *et al.*, 2004), isolated from a dinoflagellate, and an *Oceanobacter* sp. strain (AY136131; Pinhassi & Berman, 2003) isolated from the Red Sea, are from temperate regions; they showed 94 % sequence similarity to strain Gp\_4\_7.1<sup>T</sup>. Representatives of the genus *Pseudomonas* originating from Antarctic soil (GenBank accession no. AF411854) and activated sludge (AM084028) displayed a sequence similarity of only 90 %. Analysis using the ARB software and construction of a phylogenetic tree (Fig. 2) showed that strain Gp\_4\_7.1<sup>T</sup> and its nearest neighbours were affiliated with the order *Oceanospirillales*, within which strain Gp\_4\_7.1<sup>T</sup> formed an independent phylogenetic line.

It is apparent from the phenotypic and chemotaxonomic properties, as well as 16S rRNA gene sequence data, that strain Gp\_4\_7.1<sup>T</sup>, isolated from the marine sponge *Isops phlegraei* collected at the Sula Ridge off the Norwegian coast, cannot be assigned to any previously recognized bacterial genus and represents a novel species within a new genus, *Spongiispira norvegica* gen. nov., sp. nov.

### Description of *Spongiispira* gen. nov.

*Spongiispira* (Spon.gi.i.spi'ra. L. fem. n. *spongia* sponge; L. fem. n. *spira* curvature, spiral; N.L. fem. n. *Spongiispira* spiral-shaped bacterium from a sponge).

Gram-negative, spiral-shaped cells, motile by a single polar flagellum. Aerobic, chemoheterotrophic, positive for lipase activity, relatively restricted nutritional profile, not able to reduce nitrate, oxidase-positive, catalase-negative and mesophilic. Salt is essential for growth. The major cellular fatty acids are C<sub>16:1</sub>ω7 and C<sub>16:0</sub>. Belongs to the class



**Fig. 2.** Phylogenetic maximum-parsimony tree calculated from partial 16S rRNA gene sequences (1403 bp). The novel isolate and its nearest described neighbour are indicated in bold. Maximum-parsimony bootstrap values (1000 resamplings) are indicated as percentages at branch points. Bar, 1 % sequence divergence. The tree was rooted using *Thermotoga maritima* MSB8<sup>T</sup> (GenBank accession no. M21774) as an outgroup (not shown).

Gammaproteobacteria, order Oceanospirillales. The type species is *Spongiispira norvegica*.

### Description of *Spongiispira norvegica* sp. nov.

*Spongiispira norvegica* (nor.ve'gi.ca. N.L. fem. adj. *norvegica* Norwegian, referring to the collection off the Norwegian coast of the sponge from which the type strain was isolated).

Has the following characteristics in addition to those given above for the genus. Spiral cells are 2–5 µm in length and 0.3–0.4 µm in diameter. Colonies on MBA are beige and circular with a diffuse and flat shape. Temperature range for growth is 6–37 °C, with an optimum at 20–30 °C. The pH range for growth is 6.5–8.5, with an optimum at pH 7–8. Growth is observed in media with salt concentrations of 2–7 % (w/v), with an optimum of 2–3 % (w/v) artificial seawater. Does not grow with NaCl as sole salt source. Positive for esterase lipase C8, lipase C14, leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities. Arabinose can be utilized. The dominant fatty acids are C<sub>16:1</sub>ω7 (39.4 %), C<sub>16:0</sub> (29.7 %), C<sub>18:0</sub> (8.1 %), C<sub>18:1</sub>ω9 (6.9 %) and C<sub>10:0</sub> 3-OH (6.2 %).

The type strain is Gp\_4\_7.1<sup>T</sup> (=DSM 17749<sup>T</sup> =NCIMB 14401<sup>T</sup>), isolated from the marine sponge *Isops phlegraei* at the Sula Ridge near the coast of mid-Norway. The DNA G+C content of the type strain is 62.6 mol%.

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