

## *Sphingomonas jejuensis* sp. nov., Isolated from Marine Sponge *Hymeniacidon flavia*<sup>§</sup>

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A Gram-negative, non-motile, rod shaped, and orange-pigmented chemoheterotrophic bacterium, strain MS-31<sup>T</sup> was isolated from the marine sponge *Hymeniacidon flavia*, collected from near Jeju Island, Korea. The Strain MS-31<sup>T</sup> was subjected to a polyphasic taxonomic study. The phylogenetic analysis based on the 16S rRNA gene sequences revealed that the novel isolate could be affiliated within the genus *Sphingomonas*. The strain MS-31<sup>T</sup> showed 95.6% of 16S rRNA gene sequence similarity with the most closely related species *Sphingomonas koreensis* JSS26<sup>T</sup>. The DNA G+C content of the strain MS-31<sup>T</sup> was 69.4 mol%. The major isoprenoid quinone was ubiquinone 10 and predominant cellular fatty acids were summed feature 7 (comprising C<sub>18:1</sub> ω7c, C<sub>18:1</sub> ω9t and/or C<sub>18:1</sub> ω12t, 39.7%), C<sub>16:0</sub> (16.3%), C<sub>14:0</sub> 2OH (15.9%) and summed feature 3 (comprising C<sub>16:1</sub> ω7c and/or C<sub>15:0</sub> iso 2OH, 11.7%). The polar lipids were sphingoglycolipid, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and unidentified glycolipid. Based on the evidence from the polyphasic taxonomic study, the strain should be classified as a new species of the genus *Sphingomonas*. As a result, the name *Sphingomonas jejuensis* sp. nov. (type strain MS-31<sup>T</sup> =KCTC 23321<sup>T</sup> =NBRC 107775<sup>T</sup>) is proposed.

**Keywords:** marine sponge, *Sphingomonas jejuensis* sp. nov.

The genus *Sphingomonas* was initially proposed by Yabuuchi *et al.* (1990). Currently, the genus *Sphingomonas* was comprised of approximately 45 species with validly published names, including the recently described species: *Sphingomonas chagbensis* (Zhang *et al.*, 2010) and *Sphingomonas histidinilytica* (Nigam *et al.*, 2010). Members of the genus *Sphingomonas* sensu strict have been isolated from many natural sources: rhizospheres, soil, aquatic habitats and clinical material. However, the *Sphingomonas* strains dwelling in marine environments, particularly those associated with animals, were not studied much. A novel aerobic bacterium, the strain MS-31<sup>T</sup> was isolated from the marine sponge *Hymeniacidon flavia*. Polyphasic taxonomical analysis demonstrated that the strain MS-31<sup>T</sup> proposed a novel species as *Sphingomonas jejuensis* sp. nov. within the genus *Sphingomonas*.

### Materials and Methods

#### Isolation of bacterial strain and cultivation

An aerobic bacterium was isolated from the marine sponge *Hymeniacidon flavia*, obtained from the sea near Jeju Island, Republic of Korea (33°13' N, 126°33' E; depth, 20 m). The *Hymeniacidon flavia* (0.5-1 cm<sup>3</sup>) was homogenized with a glass rod in 5 ml sterile seawater. 50 μl of homogenate was spread on the medium P (Yoon *et al.*, 2007). After the cultivation at 15°C for 4 weeks, colonies were picked and

re-isolated on 1/2 strength Marine agar 2216 (MA: Difco, USA) with 1% extra NaCl. The obtained bacterial strain MS-31<sup>T</sup> was maintained and experimented on Trypticase soy agar (TSA: Difco) at 25°C. The temperature (4°C, 15°C, 20°C, 25°C, 30°C, 37°C, and 40°C) and pH (5, 6, 7, 8, 9, and 10) ranges for the growth were determined by incubating the isolate on TSA. The NaCl concentration for the growth was determined on TSA medium with NaCl (0, 0.3, 0.5, 1, 2, 3, 5, and 10% w/v).

#### Morphological, physiological and biochemical tests

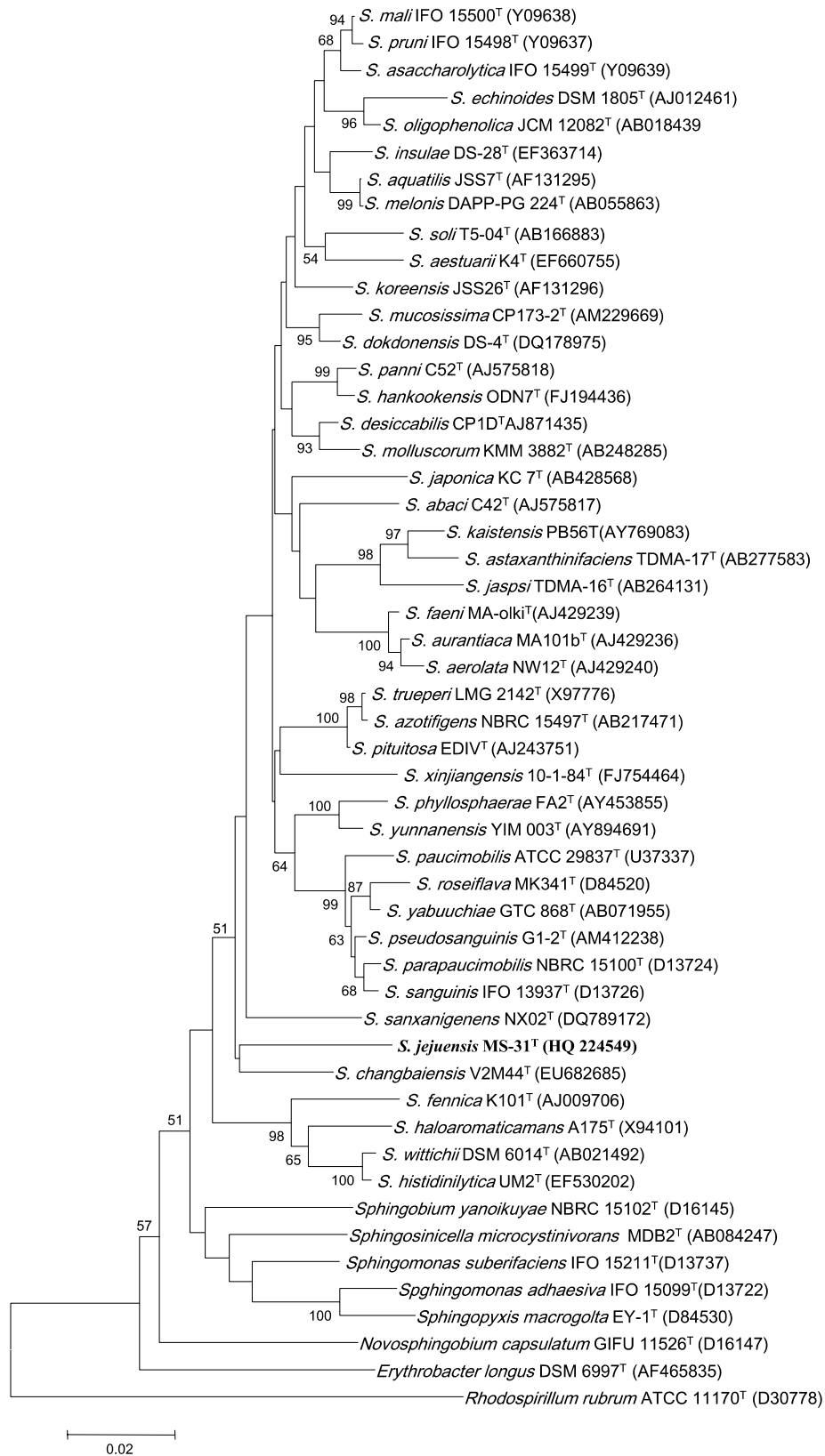
Gram-staining was performed as described by Murray *et al.* (1994). Cell morphology and motility were observed by the light microscopy (BX60; Olympus, Japan). The growth under anaerobic condition was determined after 4 weeks incubation in the AnaeroPack (Mitsubishi Gas Chemical Co., Japan) on TSA. Catalase activity was determined by the bubble formation in the 3% H<sub>2</sub>O<sub>2</sub> solution. Oxidase activity was determined by using the cytochrome oxidase test paper (Nissui Pharmaceutical Co., Japan). API 20E, API 20NE, API 50CH and API ZYM strips (bioMérieux, France) were used to determine physiological and biochemical properties of the strain MS-31<sup>T</sup>, *S. koreensis* JSS26<sup>T</sup> (KCTC 2882) and *S. dokdonensis* DS-4<sup>T</sup> (KCTC 12541). API 20E, API 20NE, API 50CH test strips were read after 4 days incubation and API ZYM test strip was read after 2 days incubation. All the incubations were done at 25°C.

#### 16S rRNA gene sequencing and phylogenetic analysis

Approximately 1,400 bp of the 16S rRNA gene was amplified from the extracted DNA of the strain MS-31<sup>T</sup> by the bacterial universal primers specific to the 16S rRNA gene: 27F and 1492R (Weisburg

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**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of MS-31<sup>T</sup> among the currently known and related species of the genus *Sphingomonas*. Numbers at nodes are bootstrap percentages derived from 1,000 replications. The sequence of *Rhodospirillum rubrum* ATCC 11170<sup>T</sup> (D30778) was used as an outgroup. Scale bar, 0.02 substitutions per nucleotide position.

*et al.*, 1991). To ascertain the phylogenetic position of the strain MS-31<sup>T</sup>, its 16S rRNA gene sequence was compared with the data from the GenBank (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>). Multiple alignments of sequences were performed by the CLUSTAL\_X (version 1.83) (Thompson *et al.*, 1997). Alignment gaps and ambiguous bases were not considered, when the 1,197 bp of the 16S rRNA gene were compared. Aligned sequences were analyzed by the MEGA 4 (Tamura *et al.*, 2007). Evolutionary distances, [distance options according to the Kimura two-parameter model (Kimura, 1983)] and clustering with the neighbour-joining (NJ: Saitou and Nei, 1987) were determined by bootstrap values based on 1,000 replications (Felsenstein, 1985). Similarities were calculated using the same software values (MEGA 4).

### Chemotaxonomic investigation

Determination of the respiratory quinone was described previously by Xie and Yokota (2003). For the analysis of fatty acid methyl esters, the Strain MS-31<sup>T</sup> was grown on TSA for a week at 25°C and *S. koreensis* JSS26<sup>T</sup> (KCTC 2882) and *S. dokdonensis* DS-4<sup>T</sup> (KCTC 12541) were grown on TSA for 3 days at 25°C. The fatty acid methyl esters were extracted and prepared according to the standard protocol, provided by the MIDI/Hewlett Packard Microbial Identification system (Sasser, 1990).

Polar lipids and sphingoglycolipids were extracted according to the procedures described by Minnikin *et al.* (1984) and Yabuuchi *et al.* (1990). They were identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata and Suzuki, 1987). Phospholipids were detected with the

Zinzadze reagent. (Dittmer and Lester *et al.*, 1964). Whole lipid profiles were detected by spraying with molybdato-phosphoric acid (10 g molybdato-phosphoric acid was diluted in 100 ml ethanol) during heating at 120-160°C (Worliczek *et al.*, 2007).

For the determination of G+C content, DNA was prepared according to the method of Marmur (1961) and DNA base composition was determined by the HPLC method of Mebash *et al.* (1989).

## Results and Discussion

### Phylogenetic analysis

Mostly completed 16S rRNA gene sequences for the strain MS-31<sup>T</sup> was determined and the FASTA search in GenBank showed that the strain belonged to the genus *Sphingomonas*. In particular, the strain MS-31<sup>T</sup> was closely related to *S. koreensis* JSS26<sup>T</sup> (95.6%), *S. dokdonensis* DS-4<sup>T</sup> (95.5%), *S. changbaiensis* V2M44<sup>T</sup> (95.4%), *S. asaccharolytica* NBRC 15499<sup>T</sup> (95.2%), *S. pituitosa* EDIV<sup>T</sup> (95.0%) and *S. molluscorum* KMM 3882<sup>T</sup> (95.0%). Based on NJ, the phylogenetic tree was generated from the comparisons of 16S rRNA gene sequences and revealed that the strain MS-31<sup>T</sup> formed a cluster with *S. changbaiensis* V2M44<sup>T</sup> with low bootstrap confidence values (<50%) (Fig. 1). The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence for the strain MS-31<sup>T</sup> is HQ 224549.

### Morphological, physiological and biochemical analysis

After incubation on TSA at 25°C for a week, cells of the

**Table 1.** Differential characteristics of strain MS-31<sup>T</sup> and related species

Strains: 1, *S. jejuensis* MS-31<sup>T</sup>; 2, *S. koreensis* JSS26<sup>T</sup>; 3, *S. dokdonensis* DS-4<sup>T</sup>; 4, *S. changbaiensis* V2M44<sup>T</sup> (Zhang *et al.*, 2010); 5, *S. asaccharolytica* NBRC 15499<sup>T</sup> (Takeuchi *et al.*, 1995); 6, *S. pituitosa* EDIV<sup>T</sup> (Denner *et al.*, 2001); 7, *S. molluscorum* KMM 3882<sup>T</sup> (Romanenko *et al.*, 2007). Y, yellow; LY, light yellow; DY, dark yellow; OR, orange; +, Positive; -, negative; (+), weakly positive; ND, not determined or no data; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SGL, sphingoglycolipid; SL, sphingolipid; PC, Phosphatidylcholine; PL, Phospholipid; PME, phosphatidylmethylethanolamine; PDE, phosphatidylmethylethanolamine. \*, data from Lee *et al.* (2001); #, data from Yoon *et al.* (2006).

Characteristic	1	2	3	4	5	6	7
Colony color	OR	Y	Y	Y	LY	DY	Y
Motility	-	+	+	+	+	+	-
Oxidase activity	-	+	+	+	ND	-	+
Nitrate reduction	+	-	-	-	-	-	+
Indole production	+	-	-	-	-	-	-
Gelatin hydrolysis	+	-	+	+	-	-	-
Aesculin hydrolysis	+	-	+	+	+	+	+
β-Galactosidase	+	+	-	-	+	+	+
Assimilation of:							
N-Acetyl-D-glucosamine	-	+	-	-	+	+	+
Adipate	-	-	+	+	-	-	-
L-Arabinose	+	-	-	-	(+)	+	+
Citrate	-	-	+	-	-	-	-
Gluconate	-	-	-	+	-	-	+
D-Glucose	+	+	+	(+)	(+)	+	+
Malate	-	+	-	(+)	-	+	-
Maltose	-	+	-	-	+	+	+
D-Mannose	-	-	-	-	(+)	+	+
DNA G+C content (mol%)	69.4	*66	#66.9	65.8	64.8	64.5	68.3
Polar lipids	DPG, PE, PG, SGL,	*SL	#DPG, PE, PG, SGL, PC, PL	DPG, PE, PG, SGL, PME	SL	PE, PME, PG, SL	DPG, PE, PC, SGL, PG, PDE

strain MS-31<sup>T</sup> had rod shaped, non-motile, 0.5-0.7 µm wide and 1.5-2.0 µm long. Spores were not formed. Cultural, physiological and biochemical characteristics of the strain MS-31<sup>T</sup> were compared to related species within the genus *Sphingomonas* (Table 1). Physiological data of API 20NE were analyzed after 4 days because we could not obtain data about MS-31<sup>T</sup> in manufacture's direction. The collected data of *S. koreensis* and *S. dokdonensis* were compared with the previous reports individually (Lee *et al.*, 2001; Yoon *et al.*, 2006). Although *S. dokdonensis* did not assimilate D-mannose in this study, it was assimilated in previous report. Other physiological data of two reference strains in Table 1 showed same results as previous reports.

The Strain MS-31<sup>T</sup> showed non-motile, negative activity of oxidase and positive activity for catalase, indole production, and nitrate reduction. Based on the comparison between the results of the strain MS-31<sup>T</sup> and other related species, it was possible to distinguish the strain MS-31<sup>T</sup> from another. No colony was observed under the anaerobic condition on TSA. Colonies occurred under the aerobic condition between 15-37°C. The optimal temperature and pH for the growth of the strain MS-31<sup>T</sup> were 20-25°C and pH 6-8.

#### Chemotaxonomic analysis

The DNA G+C content of the strain MS-31<sup>T</sup> was 69.4 mol%. It was within the range of values (60.7-69.9%) previously reported for the genus *Sphingomonas* (Zhang *et al.*, 2010). The major respiratory quinone of the strain MS-31<sup>T</sup> was ubiquinone-10, which was the same as the predominant ubiquinone of *Sphingomonas* (Yabuuchi *et al.*, 1990; Takeuchi *et al.*, 1995; Lee *et al.*, 2001; Yoon *et al.*, 2006; Zhang *et al.*, 2010). As shown in the Supplementary data Table 1, the major fatty acids of the strain MS-31<sup>T</sup> were summed feature 7 (comprising C<sub>18:1</sub> ω7c, C<sub>18:1</sub> ω9t and/or C<sub>18:1</sub> ω12t, 39.7%), C<sub>16:0</sub> (16.3%) and summed feature 3 (comprising C<sub>16:1</sub> ω7c and/or C<sub>15:0</sub> iso 2OH 11.7%). The hydroxy fatty acid was C<sub>14:0</sub> 2OH (15.9%). Summed feature 3 (11.7%) composition in the total fatty acids was distinguishable from *S. koreensis* JSS26<sup>T</sup>. Nevertheless, no 3-hydroxy fatty acids and summed featured fatty acid profile of this strain are similar with previously reported *sphingomonas* species (Yabuuchi *et al.*, 1990; Takeuchi *et al.*, 1995; Lee *et al.*, 2001; Yoon *et al.*, 2006; Zhang *et al.*, 2010).

The polar lipids profile was comprised of sphingoglycolipid, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and unidentified glycolipid. (see Supplementary data Fig. 1). Phosphatidylmonomethylethanolamine and phosphatidylcholine were not detected in the strain MS-31<sup>T</sup> and it distinguished strain MS-31<sup>T</sup> from *S. changbaiensis* (Zhang *et al.*, 2010), *S. pituitosa* (Denner *et al.*, 2001), *S. dokdonensis* (Yoon *et al.*, 2006), and *S. molluscorum* (Romansenko *et al.*, 2007).

#### Taxonomic conclusion

The strain MS-31<sup>T</sup> showed 95.6% of 16S rRNA gene sequence similarity with the *S. koreensis* JSS26<sup>T</sup>, the most related species. The characteristics of the strain showed orange color, non-motility, oxidase negative activity and positive activity of nitrate reduction, indole production, hydrolysis of gelatin and aesculin.

Based on the results of polyphasic taxonomical analysis, the

strain MS-31<sup>T</sup> was isolated from the marine sponge *Hymeniacidon flavia* and represented a novel species of the genus *Sphingomonas*. For this new species, name *Sphingomonas jejuensis* sp. nov. was proposed.

#### Description of *Sphingomonas jejuensis* sp. nov.

*Sphingomonas jejuensis* (je.ju.en'sis. N.L. fem. adj. *jejuensis* pertaining to off Jeju Island in the Republic of Korea, from where the type strain was isolated).

Cells are orange-pigmented, rod shape, Gram-negative and obligately aerobic. Cells are non-motile and non-spore-forming. Cells are 0.5-0.7 µm wide and 1.5-2.0 µm long. The temperature range for the growth is 15-37°C, with the optimal temperature range of 20-25°C. No growth is occurred above 37°C. The pH range for the growth is 6.0-9.0, with the optimal pH range of 6-8. NaCl is not required for the growth and can be tolerated up to 2% (w/v). Cells are catalase-positive but oxidase-negative. Nitrate reduction to nitrite is positive. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, α-chymotrypsin, α-glucosidase, and β-glucosidase are positive, valine arylamidase, cystine arylamidase are weakly positive but lipase (C14), acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase are negative. Agar is not hydrolyzed. Indole production is positive. Esculin is hydrolyzed. In the API 50 CH test system, acids are produced from D-fructose, arabinose, aesculin and D-turanose. All other API 50 CH test results are negative. Major cellular fatty acids are summed feature 7 (comprising C<sub>18:1</sub> ω7c, C<sub>18:1</sub> ω9t and/or C<sub>18:1</sub> ω12t, 39.7%), C<sub>16:0</sub> (16.3%), C<sub>14:0</sub> 2-OH (15.9%), summed feature 3 (comprising C<sub>16:1</sub> ω7c and/or C<sub>15:0</sub> iso 2OH 11.7%) and 11 methyl-C<sub>18:1</sub> ω7c (5.6%). The major respiratory quinone is the ubiquinone 10. The DNA G+C content of the genomic DNA of MS-31<sup>T</sup> is 69.4 mol%. The polar lipids profile is comprised of sphingoglycolipid, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and an unidentified glycolipid. The strain MS-31<sup>T</sup> (=KCTC 23321<sup>T</sup> =NBRC 107775<sup>T</sup>) was isolated from the marine sponge *Hymeniacidon flavia*.

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