Sphingomonas molluscorum sp. nov., a novel marine isolate with antimicrobial activity

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An aerobic, Gram-negative, yellow-pigmented, non-motile bacterium, designated strain KMM 3882^T, was isolated from a marine bivalve (*Anadara broughtoni*) collected from Peter the Great Bay, Sea of Japan, and was subjected to phenotypic and phylogenetic analyses. Strain KMM 3882^T was found to exert a remarkable inhibitory activity against a number of Gram-positive micro-organisms. Phylogenetic analysis based on 16S rRNA gene sequences placed strain KMM 3882^T within the genus *Sphingomonas*, as an independent lineage adjacent to *Sphingomonas dokdonensis* DS-4^T and *Sphingomonas panni* DSM 15761^T. Strain KMM 3882^T showed the highest 16S rRNA gene sequence similarity to *Sphingomonas dokdonensis* DS-4^T (97.3 %); similarities of 96.5–96.7 % were obtained with *Sphingomonas puitosa* DSM 13101^T, *Sphingomonas azotifigens* NBRC 15497^T, *Sphingomonas asaccharolytica* NBRC 15499^T, *Sphingomonas trueperi* DSM 7225^T and *Sphingomonas panni* DSM 15761^T. Chemotaxonomically, strain KMM 3882^T contained sphingoglycolipid, C_{16:0} and C_{18:1} as predominant fatty acids and 2-OH C_{14:0} as a major 2-hydroxy fatty acid, confirming the affiliation of strain KMM 3882^T with the genus *Sphingomonas*. On the basis of phylogenetic analysis, DNA–DNA hybridization and physiological and biochemical characterization, strain KMM 3882^T should be classified as representing a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas molluscorum* sp. nov. is proposed. The type strain is KMM 3882^T (= An 18^T = NRIC 0685^T = JCM 14122^T = CIP 109223^T).

Abbreviations: DPG, diphosphatidylglycerol; FAME, fatty acid methyl ester; PC, phosphatidylcholine; PDE, phosphatidylidimethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PME, phosphatidylmonomethylethanolamine; SGL, sphingoglycolipid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KMM 3882^T is AB248285.

Tables detailing the antimicrobial properties of strain KMM 3882^T and cellular fatty acid profiles of strain KMM 3882^T and the type strains of related *Sphingomonas* species, and figures showing a chromatogram of the polar lipids of strain KMM 3882^T and phylogenetic trees based on 16S rRNA gene sequences constructed by using maximum-likelihood and maximum-parsimony are available as supplementary material in IJSEM Online.

The genus *Sphingomonas* was created by Yabuuchi *et al.* (1990) and emended subsequently by Takeuchi *et al.* (2001), Yabuuchi *et al.* (2002) and Busse *et al.* (2003). According to Busse *et al.* (2003), *Sphingomonas* species comprising cluster I proposed by Takeuchi *et al.* (2001) should be considered as *Sphingomonas sensu stricto*, including the type species *Sphingomonas paucimobilis*.

During the screening of mollusc isolates for antimicrobial activity, strain KMM 3882^T was found to have antagonistic effects against a range of Gram-positive indicator bacteria and yeast, but not Gram-negative or fungal cultures. A butanol extract of cellular biomass of strain KMM 3882^T was

found to have antimicrobial properties against Gram-positive cocci and bacilli (see Supplementary Table S1 in IJSEM Online), pointing to low-molecular-weight metabolites, which could be responsible for the antagonistic activity. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that strain KMM 3882^T belongs to the genus *Sphingomonas* and may represent a novel species of this genus. Some sphingomonads have been reported to produce the anticyanobacterial compounds argimicins (Yamaguchi *et al.*, 2003) or to be antagonists of phytopathogenic fungi (Berg & Balin, 1994). It should be noted that much more is known about the ability of *Sphingomonas* strains to degrade organic substances or pollutants, and their biosynthetic production of extracellular polymers (Denner *et al.*, 2001; Balkwill *et al.*, 2003). In the present study, we report the polyphasic characterization of strain KMM 3882^T, which was isolated from a marine mollusc, *Anadara broughtoni*. On the basis of the phenotypic and molecular data obtained, *Sphingomonas molluscorum* sp. nov. is described.

Strain KMM 3882^T was isolated from a marine bivalve specimen (*Anadara broughtoni* Schrenck 1867), collected from Peter the Great Bay, the Sea of Japan, Russia, in June 2003. The mollusc specimen was carefully rinsed with sterile seawater and cut aseptically with a scalpel. An aliquot of intervalve blood liquor was spread on agar plates of seawater medium (SWM), containing (l⁻¹): 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose, 0.2 g K₂HPO₄, 0.05 g MgSO₄ and 15.0 g agar in 750 ml seawater/250 ml distilled water. The plates were incubated for 14 days at 28 °C. Strain KMM 3882^T grew aerobically on tryptic soy agar (TSA), marine agar 2216 and marine broth (all from Difco) at 28 °C. Strain KMM 3882^T was stored at -80 °C in marine broth supplemented with 30 % (v/v) glycerol. Motility was observed by using the hanging drop method. Cell morphology was examined by scanning electron microscopy of exponential phase cells grown in SWM. The Gram-reaction, oxidase and catalase reactions, production of H₂S and indole, and hydrolysis of casein, gelatin, starch, chitin, Tween 80, Tween 40 and Tween 20 were tested according to methods described by Smibert & Krieg (1994). The oxidation-fermentation medium of Leifson (1963) was used to test acid production from carbohydrates, with 1 % (w/v) of each compound being used. Growth at various temperatures, pH values and salinities, and substrate utilization were studied as described previously (Romanenko *et al.*, 2003, 2005). In addition, biochemical tests were carried out using API 20NE test kits (bioMérieux), as described by the manufacturer. API test results were read after 24 h and 2 days of incubation at 28 °C.

For fatty acid analysis, strain KMM 3882^T was grown on TSA at 28 °C for 48 h. Fatty acid methyl esters (FAMES) were obtained by using mild alkaline or acid methanolysis, as described by Rowe *et al.* (2000). The FAMES were extracted with hexane and analysed by using a GLC-MS Hewlett Packard model 6890 gas chromatograph equipped with an

HP 5 MS 5 % Phenyl Methyl Siloxane capillary column (30 m × 250 µm × 0.25 µm), connected to a Hewlett Packard model 5973 mass spectrometer. Identification of the FAMES was accomplished by using equivalent chain-length values, comparing the retention times of the samples to those of standards. Lipids were extracted by using the chloroform/methanol extraction method of Bligh & Dyer (1959). The polar lipid composition was determined as described by Denner *et al.* (2001). Yellow pigments were tested by using TLC (chloroform/methanol, 5 : 3, v/v). The visible spectrum of the acetone extract was examined with a CECIL 7250 spectrophotometer.

To investigate the production of antimicrobial compounds, strain KMM 3882^T was cultivated for 48 h at 28 °C on agar slants of SWM supplemented with 0.2 % (w/v) casein hydrolysate. The cells were harvested, suspended in distilled water and subjected to ultrasound for 2 min. The suspension obtained was extracted twice with equal volumes of n-butanol. The organic layers were evaporated to dryness and the residue was diluted in an appropriate amount of ethanol and centrifuged to remove insoluble substances. The cellular extract obtained, referred to as butanol extract, was examined for the presence of antimicrobial activity by using the agar diffusion method. To test for antimicrobial activity, 100 µl butanol extract (100 µg/100 µl, in 10 % ethanol) was placed in wells (9 mm in diameter) cut in nutrient agar plates that had been inoculated with 100 µl of an indicator culture suspension (10⁸ cells ml⁻¹). The plates were incubated at 28 °C for 3 days and examined daily. The formation of a clear inhibition zone around the wells was considered to be indicative of antimicrobial activity. Zones of inhibition were recorded by measuring the clear zone diameter in millimetres, including the 9 mm diameter of the well. All tests were performed in triplicate. The indicator strains used are listed in Supplementary Table S1 in IJSEM Online. *Enterococcus faecium* CIP 104105 and *Staphylococcus aureus* CIP 65.8^T were obtained from Collection de l'Institut Pasteur (CIP), Paris, France, and the *Bacillus* species were obtained from the All Russian Collection of Microorganisms (VKM), Pushchino, Russia.

The DNA base composition was determined as described by Marmur & Doty (1962) and Owen *et al.* (1969). DNA-DNA relatedness was measured by using the photobiotin-labelled DNA probe microplate method of Ezaki *et al.* (1989). The 16S rRNA gene sequence (1381 bp) of strain KMM 3882^T was determined and analysed as described by Shida *et al.* (1997). The sequence was compared with 16S rRNA gene sequences retrieved from the EMBL/GenBank/DBJ databases by using the FASTA program (Pearson & Lipman, 1988). Distances were calculated according to the method of Jukes & Cantor (1969). Sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) in the InforBIO software (Sugawara *et al.*, 2003) and putative variable regions, positions 70–100, 181–219, 447–487, 1004–1036, 1133–1141 and 1446–1456 (*Escherichia coli* numbering system) (Anzai *et al.*, 2000), were eliminated.

Phylogenetic trees were constructed by using maximum-likelihood and maximum-parsimony methods with the PHYLIP package (Felsenstein, 1989), and the neighbour-joining method of Saitou & Nei (1987) with the CLUSTAL W program.

Cells of strain KMM 3882^T were Gram-negative, aerobic, oxidase- and catalase-positive, yellow-pigmented and non-motile. Physiological, biochemical and chemotaxonomic characteristics of strain KMM 3882^T are given in Table 1, in Supplementary Table S2 and Fig. S1 available in IJSEM Online, and in the species description. The predominant polar lipids of strain KMM 3882^T comprised phosphatidylcholine (PC), sphingoglycolipid (SGL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG). Phosphatidylmethylethanolamine (PDE), aminolipid and two unknown lipids were also present as minor components (see Supplementary Fig. S1 in IJSEM Online). Two acetone-soluble pigments were found to have the following spectral characteristics: λ_{max} at 426 and 449 nm and a shoulder at 478 nm for the first; and λ_{max} at 451 and 479 nm and a shoulder at 426 nm for the second. The polar

Table 1. Phenotypic characteristics of *Sphingomonas moluscorum* sp. nov. and related *Sphingomonas* species

Taxa: 1, strain KMM 3882^T (data from this study); 2, *Sphingomonas pituitosa* (Denner *et al.*, 2001); 3, *Sphingomonas trueperi* (Kämpfer *et al.*, 1997); 4, *Sphingomonas panni* (Busse *et al.*, 2005); 5, *Sphingomonas azotifigens* (Xie & Yokota, 2006); 6, *Sphingomonas paucimobilis* (Yabuuchi *et al.*, 1990; Kämpfer *et al.*, 1997). +, Positive; –, negative; W, weak reaction; V, variable reaction between strains; ND, not determined. All were positive for aesculin hydrolysis and β -galactosidase production, and negative for indole production and assimilation of phenylacetate.

Characteristic	1	2	3	4	5	6
Motility	–	+	+	–	+	+
Oxidase	+	–	+	–	+	–
Nitrate reduction	–	–	+	ND	–	–
Gelatin hydrolysis	–	–	+	ND	V	+
Starch hydrolysis	–	–	–	ND	+	+
Tween 80	+	+	–	ND	+	+
Assimilation of:						
D-Glucose	+	+	+	–	+	+
L-Arabinose	+	+	+	–	+	+
D-Mannose	+	–	+	W	ND	+
Maltose	+	+	+	W	+	+
N-Acetylglucosamine	+	+	+	–	ND	+
D-Gluconate	+	+	–	–	–	–
Caprate	–	+	–	ND	ND	–
Adipate	–	+	–	–	–	–
L-Malate	–	+	+	+	+	+
Citrate	–	–	–	+	–	–
DNA G+C content (mol%)	68.3	64.5	65.6	ND	66.0–68.0	63.7

lipid composition of strain KMM 3882^T was consistent in its major components, PC, SGL, PG, PE and DPG, with those reported for the closely related species, *Sphingomonas trueperi* (Kämpfer *et al.*, 1997), *Sphingomonas paucimobilis* (Kämpfer *et al.*, 1997) and *Sphingomonas panni* (Busse *et al.*, 2005). Denner *et al.* (2001) reported for *Sphingomonas pituitosa* the presence of DPG, PE, PDE, PG, SGL and an unidentified polar lipid as major components and PC, phosphatidylmonomethylethanolamine (PME), aminophospholipid, unknown phospholipids and an unknown glycolipid as minor components. Strain KMM 3882^T could be distinguished from the above-mentioned species and from other recognized *Sphingomonas* species (Kämpfer *et al.*, 1997; Denner *et al.*, 2001; Busse *et al.*, 2003) by the presence of the minor polar lipid components of unknown lipids and aminolipid and the absence of PME and glycolipids.

The fatty acid profile of strain KMM 3882^T was characterized by the occurrence of C_{16:0} and C_{18:1} as predominant fatty acids (58.2 %), 2-OH C_{14:0} (12.8 %) as a major 2-hydroxy fatty acid and the absence of 3-hydroxy fatty acids. The fatty acid composition is given in more detail in the species description and in Supplementary Table S2 available in IJSEM Online. The cellular fatty acid profile of strain KMM 3882^T differed from those of the type strains of related *Sphingomonas* species by the presence of C_{18:2 ω 6} (8.1 %), C_{19:1} (3.9 %), C_{15:1} (1.6 %) and the abundance of C_{18:0} (9.2 %). The latter fatty acid was present only in *S. azotifigens* NBRC 15497^T (3.8 %; Xie & Yokota, 2006). Unlike *Sphingomonas pituitosa* DSM 13101^T and *Sphingomonas azotifigens* NBRC 15497^T, strain KMM 3882^T did not contain 2-OH C_{15:0}. The chemotaxonomic features of strain KMM 3882^T, including the presence of sphingoglycolipid, C_{16:0}, C_{18:1} and 2-OH C_{14:0} as major fatty acids and the absence of 3-hydroxy fatty acids are in line with the characteristic components reported for recognized species of the genus *Sphingomonas* (Kämpfer *et al.*, 1997; Takeuchi *et al.*, 2001; Busse *et al.*, 2003).

The DNA G+C content of strain KMM 3882^T was 68.3 mol%, which is within the range of values (61.6–68.0 mol%) reported for recognized *Sphingomonas* species. The value obtained is similar to those determined for recently described *Sphingomonas azotifigens* strains (66.0–68.0 mol%), but is slightly higher than the values (63.7–65.6 mol%) determined for *Sphingomonas pituitosa*, *Sphingomonas trueperi* and the type species *Sphingomonas paucimobilis* (Table 1).

On the basis of the morphological and physiological characteristics, polar lipid composition, fatty acid profile and the DNA G+C content, strain KMM 3882^T can be considered to be a member of the genus *Sphingomonas*.

Phylogenetic analysis based on 16S rRNA gene sequences positioned strain KMM 3882^T within the genus *Sphingomonas* as an independent lineage adjacent to *Sphingomonas dokdonensis* DS-4^T (Yoon *et al.*, 2006) and *Sphingomonas panni* DSM 15761^T (Busse *et al.*, 2005)

(Fig. 1). The same relationship was also evident in 16S rRNA gene sequence dendrograms based on different treeing algorithms (see Supplementary Figs S1 and S2 in IJSEM Online). Strain KMM 3882^T showed the highest 16S rRNA gene sequence similarity of 97.3 % to *Sphingomonas dokdonensis* DS-4^T; similarities of 96.5–96.7 % were obtained with *Sphingomonas pituitosa* DSM 13101^T, *Sphingomonas azotifigens* NBRC 15497^T, *Sphingomonas asaccharolytica* NBRC 15499^T, *Sphingomonas trueperi* DSM 7225^T and *Sphingomonas panni* DSM 15761^T. The 16S rRNA gene sequence similarity value of 97 % is consistent with the criterion for differentiation of bacteria at the species level given by Stackebrandt & Goebel (1994). Relatively low 16S rRNA gene sequence similarity values (96.7 % to *Sphingomonas panni* DSM 15761^T, 96.6 % to *Sphingomonas trueperi* DSM 7225^T and 96.5 % to *Sphingomonas pituitosa* DSM 13101^T, *Sphingomonas azotifigens* NBRC 15497^T and *Sphingomonas asaccharolytica* NBRC 15499^T and values below these to other *Sphingomonas* species) clearly demonstrated that strain KMM 3882^T could be regarded as representing a novel species within the genus *Sphingomonas*.

The level of DNA–DNA relatedness for strain KMM 3882^T and *Sphingomonas dokdonensis* DS-4^T was determined, because of their 16S rRNA gene sequence similarity of 97.3 %. The DNA–DNA relatedness of 28 % determined for strain KMM 3882^T and *Sphingomonas dokdonensis* DS-4^T was significantly less than 70 %, indicating that these strains belong to separate species (Wayne *et al.*, 1987).

The phylogenetic distinction of strain KMM 3882^T was supported by its phenotypic characteristics. The phenotypic features of strain KMM 3882^T and phylogenetically related species are given in Table 1. *Sphingomonas dokdonensis*

DS-4^T was not included because of its recent description (Yoon *et al.*, 2006). Strain KMM 3882^T could be distinguished from its close relatives by a combination of phenotypic features. Strain KMM 3882^T was similar to *Sphingomonas panni* in being non-motile, but differed in oxidase reaction and assimilation pattern. Strain KMM 3882^T was similar to *Sphingomonas trueperi*, *Sphingomonas paucimobilis* (Kämpfer *et al.*, 1997) and *Sphingomonas azotifigens* (Xie & Yokota, 2006) in being able to assimilate D-glucose, L-arabinose, maltose and N-acetylglucosamine, but differed from all these species in being non-motile and able to assimilate D-gluconate, and from *Sphingomonas trueperi* and *Sphingomonas paucimobilis* by not being able to hydrolyse gelatin, and from *Sphingomonas paucimobilis* and *Sphingomonas azotifigens* by not being able to hydrolyse starch.

Based on the results obtained, it is proposed that strain KMM 3882^T represents a novel species in the genus *Sphingomonas*, with the name *Sphingomonas molluscorum* sp. nov.

Description of *Sphingomonas molluscorum* sp. nov.

Sphingomonas molluscorum (mol.lus.co'rum. N.L. pl. n. *Mollusca* a zoological phylum; N.L. gen. pl. n. *molluscorum* of molluscs classified in the phylum *Mollusca*).

Cells are Gram-negative, non-sporing, non-motile, small rods (0.4–0.6 µm wide and 1.0–1.5 µm long). Form yellow-pigmented, smooth, shining and opaque colonies with regular edges, and 3–4 mm in diameter. Diffusible pigments are not produced. Grows at 7–37 °C, with optimum growth at 25–28 °C. Does not grow at 4 or 42 °C. Sodium ions are

Fig. 1. Neighbour-joining tree showing the relationships between strain KMM 3882^T and related *Sphingomonas* species based on 16S rRNA gene sequences. Numbers indicate bootstrap values greater than 700. Bar, 0.02 *K_{nucl}*. Trees generated using maximum-likelihood and maximum-parsimony are available as Supplementary Figs S2 and S3 in IJSEM Online.

not required for growth. Grows in 0–4% NaCl. Oxidase-, catalase- and β -galactosidase-positive. Indole, H₂S, urease, arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase and phenylalanine deaminase are not produced. Casein, gelatin, starch and chitin are not hydrolysed. Hydrolysis of Tween 80, Tween 40 and Tween 20 is positive. Production of acid from arabinose, galactose and D-xylose is weakly positive. Acid is not produced from D-glucose, rhamnose, maltose, sucrose, lactose, inositol, mannitol or glycerol. Other physiological and biochemical characteristics are given in Table 1. In addition, in API tests, assimilation of mannitol is negative. According to conventional methods, nitrate reduction and utilization of sucrose, inositol, sorbitol and mannitol are negative; utilization of D-glucose, L-arabinose, lactose, D-mannose and malonate is positive. Predominant polar lipids are PC, SGL, PG, PE and DPG. PDE, aminolipid and two unknown lipids are present as minor components. Two acetone-soluble pigments are characterized by $\lambda_{\max}$ at 426 and 449 nm and a shoulder at 478 nm, and $\lambda_{\max}$ at 451 and 479 nm and a shoulder at 426 nm. Contains C_{16:0} (24.0%), C_{18:1 ω 9} (16.5%) and C_{18:1 ω 7c} (17.7%) as major fatty acids and 2-OH C_{14:0} (12.8%) as a major 2-hydroxy fatty acid; C_{15:0} (1.7%); C_{15:1} (1.6%), C_{16:1} (2.1%), C_{17:1} (2.5%), C_{18:2 ω 6} (8.1%), C_{18:0} (9.2%) and C_{19:1} (3.9%) are also present.

The DNA G+C content of the type strain is 68.3 mol% (determined by using the thermal denaturation method). The type strain is KMM 3882^T (=An 18^T=NRIC 0685^T=JCM 14122^T=CIP 109223^T), which was isolated from a marine bivalve (*Anadara broughtoni*), collected from Peter the Great Bay, The Sea of Japan, Russia.

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