

Parasphingopyxis algicola sp. nov., isolated from a marine red alga *Asparagopsis taxiformis* and emended description of the genus *Parasphingopyxis* Uchida *et al.* 2012

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Abstract

An aerobic, Gram-stain-negative, yellow-pigmented bacterium, designated strain ATAX6-5^T, was isolated from a marine red alga, *Asparagopsistaxiformis*, in South Korea. Cells were non-motile rods showing catalase- and oxidase-positive reactions. Growth of strain ATAX6-5^T was observed at 5–35 °C (optimum, 30 °C), at pH 6.0–9.5 (optimum, pH 7.0) and in the presence of 0–6.0 % (w/v) NaCl (optimum, 2 %). Ubiquinone-10 was detected as the sole isoprenoid quinone and $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{17:1}$ $\omega6c$ were identified as the major cellular fatty acids. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, sphingoglycolipid, phosphatidylcholine, an unknown phospholipid and four unknown glycolipids were detected as polar lipids. The G+C content of the genomic DNA was 60.4 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain ATAX6-5^T formed a tight phylogenic lineage with *Parasphingopyxis lamellibrachiae* JAMH 0132^T with a 100 % bootstrap value. Strain ATAX6-5^T was most closely related to *P. lamellibrachiae* JAMH 0132^T with a 96.9 % 16S rRNA gene sequence similarity. Based on phenotypic, chemotaxonomic and molecular features, strain ATAX6-5^T clearly represents a novel species of the genus *Parasphingopyxis*, for which the name *Parasphingopyxis algicola* sp. nov. is proposed. The type strain is ATAX6-5^T (=KACC 18993^T=JCM 31719^T). An emended description of the genus *Parasphingopyxis* is also proposed.

The genus Parasphingopyxis, belonging to the family Sphingomonadaceae of the class Alphaproteobacteria, was first proposed by Uchida et al. [1]. At the time of writing, the genus Parasphingopyxis comprises only one species with a validly published name, Parasphingopyxis lamellibrachiae, which was isolated from a marine annelid worm. Uchida et al. [1] described that cells of the genus Parasphingopyxis are Gram-stain-negative, aerobic, catalase- and oxidase-positive, yellow-pigmented, slightly halophilic motile rods. In addition, they described that cells of the genus Parasphingopyxis contain ubiquinone-10 as the predominant respiratory quinone, $C_{18:1}\omega7c$ and $C_{16:0}$ as the major fatty acids and phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine and sphingoglycolipid as the major polar lipids. Algal spheres (the surface or inside of algal cells) are unique bacterial habitats distinct from the ambient water and where various algae-bacteria interactions, such as symbiosis, competition, commensalism and mutualism, and ecologically important metabolic processes, such as nitrogen fixation, photosynthesis, nutrient remineralization, algae growth enhancement and various chemical defences are demonstrated [2, 3]. Algal-associated bacteria are attracting a lot of attention in the effort to understand these algae-bacteria interactions and they should be considered in algal culture systems [4]. In addition, it has been reported that algal-associated bacteria produce various compounds that are useful as food additives, pharmaceuticals and industrial chemicals [5]. Therefore, the isolation of numerous algal-associated bacteria from algal spheres has been attempted [6–10] and in this study, we isolated a presumably novel species belonging to the genus *Parasphingopyxis*, designated strain ATAX6-5^T, from a marine micro-red alga.

Strain ATAX6-5^T was isolated from the culture of a marine red alga, *Asparagopsis taxiformis*. Briefly, *A. taxiformis*, which was isolated from the Yellow (West) Sea of South Korea (36° 54′ 15.9″ N 126° 11′ 52.8″ E), was cultivated in sterile L1 medium (NCMA, USA; [11]) at 25 °C for 4 weeks under light–dark conditions. Cells of *A. taxiformis* were macerated using a homogenizer for 1 min and then serially diluted in artificial seawater (ASW; 20 g NaCl, 2.9 g MgSO₄, 4.53 g MgCl₂• 6H₂O, 0.64 g KCl, 1.75 g CaCl₂• 2H₂O per

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Abbreviations: DDH, DNA–DNA hybridization; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; Q-10, ubiquinone.

The GenBank accession number for the 16S rRNA gene sequence of strain ATAX6-5^T is KY200670.

One supplementary table and two supplementary figures are available with the online Supplementary Material.

litre). The aliquots of each serial dilution were spread on 1/5 strength marine agar 2216 (MA; BD) supplemented with NaCl to a final concentration of 2 % (w/v) and incubated aerobically at 25 °C for 3 days. The 16S rRNA genes of colonies grown on the 1/5 strength MA were PCR-amplified using the universal primers, F1 and R13, and doubledigested with HaeIII and HhaI, as described previously [12], and the representative PCR amplicons showing distinct fragment patterns were partially sequenced using the universal primer 340F (5'-CCT ACG GGA GGC AGC AG-3'). The resulting 16S rRNA gene sequences were compared with those of all reported type strains using the Nucleotide Similarity Search program in the EzTaxon-e server (http:// ezbiocloud.net/identify; [13]). From the analysis, a presumably novel strain belonging to the genus Parasphingopyxis, designated ATAX6-5^T, was selected for further phenotypic and phylogenetic analyses. Strain ATAX6-5^T was routinely cultured aerobically on MA at 30 °C for 2 days, except where indicated, and stored at -80 °C in marine broth (MB; BD) supplemented with 15% (v/v) glycerol for a long-term preservation. Parasphingopyxis lamellibrachiae JCM 15549^T was purchased from a culture collection centre (Japan Collection of Microorganisms, Japan) as a reference strain for the comparison of phenotypic properties and fatty acid compositions.

The 16S rRNA gene amplicon of strain ATAX6-5^T that was PCR-amplified by 27F and 1492R primers was further sequenced using the universal primers 340F (5'-CCT ACG GGA GGC AGC AG-3'), 518R (5'-ATT ACC GCG GCT GCT GG-3') and 805F (5'-GAT TAG ATA CCC TGG TAG TC-3') at Macrogen (Korea) to obtain an almost-complete 16S rRNA gene sequence (1414 nucleotides). The 16S rRNA gene sequences of strain ATAX6-5^T and closely related type strains were aligned using the fast secondary structure aware Infernal aligner by the Ribosomal Database Project [14]. Phylogenetic relationships between strain ATAX6-5^T and closely related type strains were inferred using the DNADIST and DNAPARS programs based on the neighbour-joining (NJ) algorithm with the Kimura two-parameter model and the maximum-parsimony (MP) algorithm through a heuristic search, respectively, in the PHYLIP software (version 3.695, [15]) and their tree topologies were evaluated through bootstrap analyses based on 1000 resamplings. A maximum-likelihood (ML) analysis with bootstrap values was conducted using RAxML-HPC BlackBox (version 8.2.4) with a GTRCAT model of evolution available from the Cyber-Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org; [16]).

A phylogenetic analysis based on the NJ algorithm showed that strain ATAX6-5^T formed a tight phylogenic lineage with *P. lamellibrachiae* JAMH 0132^{T} within the family *Sphingomonadaceae* with a 100% bootstrap value (Fig. 1). Phylogenetic trees reconstructed using the MP and ML algorithms also supported that strain ATAX6-5^T formed a tight phylogenic lineage with *P. lamellibrachiae* JAMH 0132^{T} . However, the phylogenetic analyses showed that

strain ATAX6-5^T was phylogenetically distinct from other related genera including Sphingorhabdus, Sphingopyxis, Novosphingobium and Rhizorhapis. Comparative analysis based on the 16S rRNA gene sequences revealed that strain ATAX6-5^T was most closely related to *P. lamellibrachiae* JAMH 0132^T with a 96.9% sequence similarity, but sequence similarities between strain ATAX6-5^T and the type strains of other genera were <95.0 %. Recently, 98.65-98.7 % of 16S rRNA gene sequence similarity between two strains has been suggested as an alternative threshold to avoid DNA-DNA hybridization (DDH) for the new bacterial species delineation because 98.65-98.7 % of 16S rRNA gene sequence similarity equates to 70% relatedness of genomic DNA [17-19]. The 16S rRNA gene sequence similarity between strain ATAX6-5^T and P. lamellibrachiae JAMH 0132^T was much lower than the new threshold for bacterial species delineation, suggesting that strain ATAX6- 5^{T} can represent a novel species of the genus *Parasphingo*pyxis without performing DDH.

Growth of strain ATAX6-5^T was assessed at 30 °C for 2 days on MA, R2A agar (BD), Luria-Bertani (LB; MP Biomedicals) agar, nutrient agar (NA; BD) and triptic soy agar (TSA; BD), which were supplemented with NaCl to final concentrations of approximately 2%. Growth of strain ATAX6-5^T was tested on MA at different temperatures (0-45°C at 5°C intervals) and in MB at different pH values (5.0-10.0 at 0.5 pH unit intervals) for 2 days. MB with pH values below 8.0 and pH 8.0-10.0 was prepared by using Na₂HPO₄-NaH₂PO₄ and Tris-HCl buffers, respectively, and the pH values were adjusted again if necessary after sterilization (121 °C for 15 min). Growth at different NaCl concentrations (0-10% at 1% intervals) was assessed in MB, which was prepared in the laboratory according to the BD formula, for 2 days. Gram staining was tested using the Gram stain kit (bioMérieux), according to the manufacturer's instructions. Anaerobic growth was assessed on MA at 30 °C for 21 days under the anaerobic condition (with 4-10% CO₂) using the GasPak Plus system (BBL). Cell morphology and motility of strain ATAX6-5^T were investigated using transmission electron microscopy (JEM-1010, JEOL) and phasecontrast microscopy with cells grown in MB at 30 °C for 2 days. Catalase and oxidase activities of strain ATAX6-5^T were tested by the production of oxygen bubbles in 3% (v/v) aqueous hydrogen peroxide solution and the oxidation of 1% (w/v) tetramethyl-p-phenylenediamine (Merck), respectively [20]. The following properties of strain ATAX6-5^T and the type strain of *P. lamellibrachiae* (JCM 15549^T) were investigated in parallel under the same conditions at 40 °C. Hydrolysis of Tween 20, Tween 80, casein, starch, tyrosine and aesculin was checked on MA, according to the methods described previously [20, 21]. Additional enzymatic activities, biochemical features and carbon compound oxidations of strain ATAX6-5^T were tested using the API ZYM and API 20NE kits (bioMérieux) and the GN2 MicroPlate system (Biolog), respectively, according to the instructions of the manufacturers, except that resuspended cells in artificial seawater were used as cell inocula.





Strain ATAX6-5^T grew well on MA and R2A agar containing 2 % NaCl, but did not grew on LB agar and NA containing 2% NaCl. Cells were Gram-stain-negative, non-motile rods of approximately 0.4-0.5 µm in width and 1.0-2.2 µm in length (Fig. S1, available with the online Supplementary Material). Anaerobic growth was not observed after 21 days of incubation on MA at 30 °C. On the Biolog GN2 Micro-Plate, strain ATAX6-5^T oxidized Tween 40, Tween 80, Nacetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, L-arabinose, *i*-erythritol, D-fructose, L-fructose, D-galactose, gentiobiose, α -D-glucose, *m*-inositol, maltose, D-mannitol, D-mannose, methyl β -D-glucoside, D-psicose, D-sorbitol, sucrose, trehalose, xylitol, pyruvic acid methyl ester, cis-aconitic acid, citric acid, D-galacturonic acid, D-gluconic acid, p-hydroxy phenylacetic acid, D,L-lactic acid, quinic acid, succinic acid, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, Lproline, D-serine, L-serine, γ -aminobutyric acid, urocanic acid, inosine, uridine, thymidine, glycerol, D,L- α -glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate, glycogen, lactose, lactulose, succinic acid mono-methyl ester, turanose, D-glucuronic acid, α -hydroxybutylic acid, β -hydroxybutylic acid and L-threonine, but did not oxidize other carbon compounds on the Biolog GN2 MicroPlate. Phenotypic characteristics of strain ATAX6-5^T are presented in the species description and compared with those of the type strain of *P. lamellibrachiae* in Tables 1 and S1. Most of the characteristics of strain ATAX6-5^T, such as nitrate reduction, activity of oxidase, catalase, urease and alkaline phosphatase, and indole production were in good agreement with those of *P. lamellibrachiae*, whereas some other phenotypic properties such as NaCl growth range, tyrosine hydrolysis and activity of α -chymotrypsin and cystine arylamidase differentiated strain ATAX6-5^T from *P. lamellibrachiae* (Table 1).

The isoprenoid quinone of strain ATAX6-5^T was analysed by using a high-performance liquid chromatography system (model LC-20A, Shimadzu) equipped with a reversed-phase column (250 Í 4.6 mm, Kromasil, Akzo Nobel) and a diode array detector (SPD-M20A, Shimadzu) using methanol-isopropanol (2:1, v/v) as an eluent (1 ml min⁻¹), as described previously [22]. For the cellular fatty acid analysis, strain ATAX6-5^T and the type strain of *P. lamellibrachiae* were cultivated in MA broth at 30 °C and microbial cells were harvested at the same growth phase (exponential phase, **Table 1.** Phenotypic comparisons of strain ATAX6-5^T and the type strain of *Parasphingopyxis lamellibrachiae*

Taxa: 1, strain ATAX6-5^T; 2, *P. lamellibrachiae* JCM 15549^T [1]. All strains were positive for the following characteristics: nitrate reduction*, activity* of oxidase, catalase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase and assimilation* of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, maltose, potassium gluconate, capric acid, malic acid and citric acid. All strains were negative for the following characteristics: Gram-staining, activity* of arginine dihydrolase, urease, protease, trypsin, α -galactosidase, β -glucosaminidase, α -mannosidase and α -fucosidase, indole production*, fermentation of glucose, hydrolysis* of starch, gelatin, aesculin, casein, Tween 20 and Tween 80 and assimilation* of adipic acid and phenylacetic acid. +, Positive; –, negative.

Characteristics	1	2		
Growth at:				
Temperature (optimum, °C)	5-35 (30)	5-36 (28-30)		
pH (optimum)	6.0-9.5 (7.0)	6.0-9.5 (6.5-7.0)		
NaCl (optimum, %)	0-6 (2)	0.5-5 (2)		
Hydrolysis of*:				
Tyrosine	+	-		
Enzyme activity (API ZYM) of*:				
lpha-Chymotrypsin	+	-		
Cystine arylamidase	-	+		
DNA G+C content (mol%)	60.4	60.1		
Polar lipids†	DPG, PE, PG, PC, SGL, 4 GLs, PL	DPG, PE, PG, PC, SGL, GL, L,		

*These analyses were conducted under the same conditions in this study.

†DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine; SGL, sphingoglycolipid; PL, unknown phospholipid; GL, unknown glycolipid; L, unknown lipid.

optical density=0.8 at 600 nm). The fatty acids were saponified, methylated and extracted using the standard MIDI protocol. The fatty acid methyl esters were analysed by gas chromatography (Hewlett Packard 6890) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock version 6.0B; [23]). The DNA G+C content of strain ATAX6-5^T was determined by a fluorometric method [24] using SYBR Green I and a real-time PCR thermocycler (Bio-Rad). The polar lipids of strain ATAX6-5^T were analysed by thin-layer chromatography using cells harvested during the exponential growth phase, according to the method described previously [25].

The only respiratory lipoquinone detected in strain ATAX6-5^T was ubiquinone-10 (Q-10), which was the same as that in *P. lamellibrachiae*. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, sphingoglyco-lipid, phosphatidylcholine, an unknown phospholipid and four unknown glycolipids were identified from strain ATAX6-5^T (Fig. S2), which was in relatively good

agreement with the results for *P. lamellibrachiae* (Table 1). The major cellular fatty acids of strain ATAX6-5^T (>5%) were $C_{18:1}\omega7c$ (54.7%), $C_{16:0}$ (17.5%) and $C_{17:1}\omega6c$ (10.0%). Although the overall fatty acid profile of strain ATAX6-5^T was similar to that of *P. lamellibrachiae*, there were some differences in the proportions of some fatty acid components, as shown in Table 2. The DNA G+C content of strain ATAX6-5^T was 60.4 mol%, which was similar to that of *P. lamellibrachiae* (60.1 mol%) (Table 1). In conclusion, the phenotypic and chemotaxonomic features of strain ATAX6-5^T and the phylogenetic inference support its assignment to a novel species of the genus *Parasphingopyxis*, for which the name *Parasphingopyxis algicola* sp. nov. is proposed.

DESCRIPTION OF PARASPHINGOPYXIS ALGICOLA SP. NOV.

Parasphingopyxis algicola (al.gi'co.la. L. fem. n. alga a seaweed; L. suffix. -cola, (from L. masc. or fem. n. incola) a dweller; N.L. fem. n. algicola an alga dweller).

Cells are Gram-stain-negative, strictly aerobic, non-motile rods. Colonies on MA are yellow, round and convex. Growth occurs at 5-35 °C (optimum, 30 °C), at pH 6.0–9.5 (optimum, pH 7.0) and in the presence of 0–6 % (w/v) NaCl (optimum, 2%). Positive for oxidase and catalase. Reduces nitrate to nitrite, but does not produce nitrogen gas. Hydrolyses tyrosine, but not casein, aesculin, Tween 20 and 80,

Table 2. Cellular fatty acid compositions (%) of strain $ATAX6-5^{T}$ and the type strain of *Parasphingopyxis lamellibrachiae*

Taxa: 1, strain ATAX6-5^T; 2, *P. lamellibrachiae* JCM 15549^T. All data were from this study and the compositions were expressed as percentages of the total fatty acids. Major fatty acid components (>5.0%) are highlighted in bold. Symbols: TR, trace amount (<0.5%); –, not detected.

Fatty acid	1	2
Saturated fatty acid:		
C _{16:0}	17.5	22.2
C _{17:0}	1.7	1.1
Unsaturated fatty acid:		
$C_{17:1}\omega 6c$	10.0	6.5
$C_{17:1}\omega 8c$	1.5	1.8
$C_{18:1}\omega 7c$	54.7	50.9
$C_{18:1} \omega 7c$ 11-methyl	2.0	7.7
Hydroxyl fatty acid:		
C _{16:0} 2-OH	3.1	4.7
Summed features:*		
3	1.0	tr
4	2.0	-
7	1.8	2.9

*Summed features represent groups of two or three fatty acids that cannot be separated by gas-liquid chromatography with the MIDI system. Summed feature 3, $C_{16:1}\omega 6c$ and/or $C_{16:1}\omega 7c$; summed feature 4, $C_{17:1}$ iso I and/or $C_{17:1}$ anteiso B; summed feature 7, unknown fatty acid (ECL 18.846), $C_{19:1}\omega 6c$ and/or cyclo $C_{19:1}\omega 10c$.

gelatin, and starch. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase and α -chymotrypsin activities are positive. Negative for indole production and fermentation of glucose. Arginine dihydrolase, urease, protease, trypsin, α -galactosidase, β galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase and cystine arylamidase activities are negative. Assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, malic acid and citric acid is positive, but assimilation of adipic acid and phenylacetic acid is negative. The only isoprenoid quinone is Q-10. The major cellular fatty acids are $C_{16:0}$, $C_{17:1}\omega 6c$ and $C_{18:1}\omega 7c$. Diphosphatidylglyphosphatidylethanolamine, phosphatidylglycerol, cerol, sphingoglycolipid, phosphatidylcholine, an unknown phospholipid and four unknown glycolipids are identified. The DNA G+C content of the type strain is 60.4 mol%. The type strain is ATAX6-5^T (=KACC 18993^T=JCM 31719^T), isolated from a marine red alga, Asparagopsis taxiformis, in South Korea.

EMENDED DESCRIPTION OF THE GENUS PARASPHINGOPYXIS UCHIDA ET AL. 2012

This description is based on that given by Uchida *et al.* [1] with the following amendments. Cells are motile or nonmotile and grow in the range of 0-6% (w/v) NaCl. The major cellular fatty acids are $C_{18:1}\omega7c$, $C_{16:0}$ and $C_{17:1}\omega6c$. $C_{16:0}$ 2-OH is present as a hydroxyl fatty acid.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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