Glaciecola mesophila sp. nov., a novel marine agar-digesting bacterium

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Alteromonas-like strains KMM 241^T and KMM 642, isolated from marine invertebrate specimens, were investigated to clarify their taxonomic position. The novel isolates were aerobic, Gram-negative, motile, slightly halophilic and heterotrophic and hydrolysed polysaccharides. They did not hydrolyse urea, gelatin or casein and produced acid weakly from carbohydrates. The DNA G + C content ranged between 44.6 and 44.8 mol%. DNA–DNA similarity between the two strains was 71 %. Comparison of the 16S rRNA gene sequence of strain KMM 241^T revealed 94.5–94.8 % similarity to *Glaciecola* species. The novel strains shared several phenotypic and physiological properties with members of *Glaciecola*, but they differed in their lack of pigment production, their minimal and maximal growth temperatures and their ability to hydrolyse agar and carrageenan and in the utilization of organic compounds. On the basis of phenotypic and physiological characteristics as well as phylogenetic analysis, the isolates should be assigned to a novel species, *Glaciecola mesophila* sp. nov. The type strain is strain KMM 241^T (= DSM 15026^T).

The genus *Glaciecola*, described to accommodate aerobic, psychrophilic, halophilic, pigmented bacteria, comprises two species, *Glaciecola punicea* and *Glaciecola pallidula* (Bowman *et al.*, 1998). Members of *Glaciecola* have been isolated from sea-ice diatom assemblage samples collected from coastal areas of eastern Antarctica and seem to be restricted to sea-ice habitats (Bowman *et al.*, 1998). The genus *Glaciecola* is phylogenetically closely related to *Alteromonas macleodii* within the γ -subclass of *Proteobacteria*. *Alteromonas macleodii* was originally described by Baumann *et al.* (1972) and the genus was later emended (Gauthier *et al.*, 1995).

Strain KMM 241^T was isolated from internal liquor of a specimen of the ascidian *Halocynthia aurantium*, collected from coastal sea water at a depth of 5 m in Troitsa Bay, Peter the Great Bay, Sea of Japan, Russia, in May 1990. Strain KMM 642 was isolated from tissue of the sponge *Plocamia*

sp., at a depth of 350 m near the Komandorskie Islands, east of Kamchatka, in August 1991. A homogenate of the internal tissue was diluted aseptically in sterile sea water. Aliquots of internal liquor and diluted homogenate were spread on sea-water medium (SWM) agar plates (1⁻¹: 5.0 g peptone, 2.5 g yeast extract, 1.0 g glucose, 0.2 g K₂HPO₄, 0.05 g MgSO₄, 500 ml seawater, 500 ml distilled water, 15.0 agar) and incubated for 7 days at 28 °C. Bacteria were stored at –80 °C in 30 % (v/v) glycerol. Strains KMM 241^T and KMM 642 have been deposited in the Collection of Marine Micro-organisms (KMM), Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia. The strains were routinely grown on Marine 2216 agar (MA, Difco), Marine broth (MB, Difco) and SWM at 7–30 °C.

Gram-reaction, oxidase, catalase and production of amylase, caseinase, DNase, gelatinase and lipase (Tween 80) were tested according to the methods described by Baumann *et al.* (1984) and Smibert & Krieg (1994), using MA or SWM as the basal medium. Hydrolysis of κ -carrageenan was determined as described by Yaphe & Baxter (1955). Growth at different temperatures (4–40 °C) and pH values (5.0–10.0) was tested using MA and MB;

Abbreviations: MA, Marine 2216 agar; MB, Marine 2216 broth; SWM, sea-water medium.

The GenBank/EMBL accession number for the 16S rDNA sequence of strain KMM 241^T is AJ488501.

sodium ion requirement and tolerance of various NaCl concentrations (0–12 % NaCl) were assessed using SWM prepared from the artificial sea-water base supplemented with the appropriate amount of NaCl. Motility was examined by the hanging-drop method. Leifson's medium was used for testing acid production from 1 % (w/v) carbohydrates (Leifson, 1963).

Additional biochemical tests using the API 20NE test kit (bioMérieux) and the Biolog GN MicroPlate method were performed as described by the manufacturers, except that strains were suspended in 3 or 2.5 % NaCl. Cell morphology was examined by transmission electron microscopy from exponential-phase cells grown in MB. The cells were fixed with 1 % (v/v) glutaraldehyde and negatively stained with 4 % (w/v) aqueous uranyl acetate and carbon-film. Samples were examined by a Zeiss TEM910 transmission electron microscope at an acceleration voltage of 80 kV at calibrated magnifications. For determination of cellular fatty acid composition, strains KMM 241^T, KMM 642 and *A. macleodii* ATCC 27126^T were grown on MA at 15 and 28 °C for 2 days. Whole-cell fatty acids and phospholipids were examined according to the procedures described by Svetashev *et al.* (1995) and Ivanova *et al.* (2000). DNA base composition was determined as described by Marmur & Doty (1962) and Owen *et al.* (1969). Optimal DNA–DNA relatedness was measured spectrophotometrically in 2 × SSC with 10 % DMSO at 68 °C, using the initial reassociation rate kinetic procedure (De Ley *et al.*, 1970). Extraction of genomic DNA, PCR-mediated amplification of the 16S rDNA and sequencing of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced directly using the *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. An Applied Biosystems 310 DNA Genetic Analyzer was used for electrophoresis of the sequence reaction products. The 16S rDNA sequence of KMM 241^T was compared with nucleotide sequences of the closest known relatives that were retrieved from the GenBank/EMBL databases. Phylogenetic dendrograms were reconstructed according to the method of De Soete (1983) and the neighbour-joining and maximum-likelihood methods (Felsenstein, 1993).

Cultural properties, cell morphology (Fig. 1), motility and the results of some basic physiological tests of strains KMM 241^T and KMM 642 are described in the species description below. Cells were motile with a single unsheathed polar flagellum. Strains required sodium ions for growth and grew in 1–8 % NaCl; they grew at 7–35 °C but not at 4 or above 37 °C. Respective optima are indicated in the species description. Phenotypic properties of the novel strains and some reference organisms are compared in Table 1. Strains KMM 241^T and KMM 642 differ from each other in acid production and utilization of some carbohydrates. Clear differences were observed between the novel isolates and the type strains of *Glaciecola* and *Alteromonas*

Fig. 1. Transmission electron micrographs of negatively stained cells of strains KMM 241^T (a) and KMM 642 (b). Bars, 1 μm.

species in the patterns of utilization of carbohydrates and amino acids.

The strains possessed similar whole-cell fatty acid profiles and the dominant fatty acids, when grown at 28 °C, were C_{16:0} (33.0–40.6 %), C_{16:1ω7c} (35.4–43.0 %) and C_{18:1ω7c} (5.2–10.1 %) (Table 2). These profiles are similar to those of *Alteromonas* and *Glaciecola* strains, but the latter differ in the predominance of monounsaturated fatty acids over saturated fatty acids (Bowman *et al.*, 1998). It should be noted that the fatty acid composition of strains KMM 241^T and KMM 642 depended on the growth temperature. On decreasing the growth temperature from 28 to 15 °C, the proportions of C_{17:1ω8c} and C_{18:1ω7c} increased from 3.2–2.8 to 6–8 % and from 5 to 13 % (for strain KMM

Table 1. Phenotypic characteristics of strains KMM 241^T and KMM 642, *Glaciecola* species and *A. macleodii*

Strains/species: 1, strains KMM 241^T and KMM 642; 2, *G. punicea* (data from Bowman *et al.*, 1998); 3, *G. pallidula* (Bowman *et al.*, 1998); 4, *A. macleodii* (Baumann *et al.*, 1984, unless indicated otherwise). Reactions are scored as: +, positive; –, negative; V+, variable between strains, type strain positive; V–, variable between strains, type strain negative; W, weak or delayed; ND, not determined. All strains were positive for the following tests: motility, sodium ion requirement for growth, oxidase, catalase, growth at 7–20 °C and growth in 1–6 % NaCl. All strains were negative for indole reaction, nitrate reduction, denitrification, arginine dihydrolase, chitin hydrolysis and utilization of L-arabinose, citrate, phenylacetate, adipate, L-phenylalanine, L-histidine, L-ornithine, L-threonine and putrescine.

Property	1*	2	3	4
Flagellar arrangement	Polar, 1	ND	ND	Polar, 1
Filaments present	–	+	+	–
Pigmentation	–	Pink-red	Pale pink	–
Growth at:				
0 and 4 °C	–	+	+	–
25 °C	+	+	–	+
35 °C	+	–	–	+
37 °C	–	–	–	+
40 °C	–	–	–	V+
Growth in 10 % NaCl	–	–	–	+†
Hydrolysis of:				
Urea	–	W	–	–
Gelatin, casein	–	–	–	+
Aesculin	W	V+	–	ND
Starch	+	–	V+	+
Agar, carrageenan	+	–	–	–
Tween 80	+	V–	+	+
DNA	W	–	–	+†
Acid from:				
D-Glucose	W	W	W	+†
Sucrose	W, (–)	–	–	+†
D-Galactose	–, (W)	W	W	–†
Maltose	–	–	W	+†
Glycerol	–	W	W	–†
Utilization of:				
D-Glucose, sucrose, maltose	+	–	–	+
D-Galactose, cellobiose, D-fructose, D-trehalose	+, (–)	–	–	+
D-Mannose	W, (–)	–	–	–
D-Mannitol	+	–	–	V+
Glycerol, acetate, pyruvate	–	–	+	+
N-Acetylglucosamine	–	–	–	V+
Glycogen, dextran	+	–	+	ND
DL-Lactate	–	–	+	V+
D-Gluconate, propionate	–	–	–	+
Caprate	–	ND	ND	+
L-Glutamate	–	–	+	ND
α-Glycerophosphate	W, (–)	V+	+	ND
Succinate, fumarate, L-malate, L-proline	–	+	–	–
L-Serine	+, (–)	–	–	V–
L-Tyrosine	–	V+	–	+
DNA G+C content (mol%)	44·8, (44·6)	44–46	40	44–47

*Strains KMM 241^T and KMM 642 were positive for acid production from D-fructose and D-mannose (weakly), ONPG test, α-galactosidase, α-cyclodextrin and glycyl L-glutamic acid utilization. Strain KMM 241^T was positive for melibiose, β-hydroxybutyric acid and L-aspartic acid utilization; strain KMM 642 was positive for acid production from xylose (weakly) and L-alanyl glycine utilization. Both strains were negative for H₂S production and utilization of lactose, D-sorbitol, myo-inositol, adonitol, malonate, D-glucuronate, isobutyrate, valerate, isovalerate, caprylate, L-asparagine, hydroxy-L-proline and L-leucine. Where different reactions are shown, the reaction of strain KMM 642 is given in parentheses.

†Data obtained in this study.

Table 2. Fatty acid composition of strains KMM 241^T and KMM 642 and the type strains of *Glaciecola* species and *A. macleodii*

Values are percentages of total fatty acids. Data for *Glaciecola* species were taken from Bowman *et al.* (1998). The following fatty acids were detected in some or all strains at <1%: 12:1, 13:0, 13:1, 13:0i, 14:0i, 15:0i, 14:1 ω 5c, 15:0a, 15:1 ω 6c, 17:0i, 12:0-3OH, 18:0i, 18:1 ω 11c and 18:1 ω 9c. ND, Not detected.

Fatty acid	KMM 241 ^T		KMM 642		<i>A. macleodii</i> ATCC 27126 ^T		<i>G. punicea</i> ACAM 611 ^T	<i>G. pallidula</i> ACAM 615 ^T
	15 °C	28 °C	15 °C	28 °C	15 °C	28 °C		
12:0	1.3	2.6	1.9	3.0	2.1	2.6	ND	ND
14:0	1.4	1.2	1.0	0.8	1.0	1.0	1.2	3.6
14:1 ω 7c	0.2	0.3	0.1	0.4	0.9	0.3	0.5	3.7
15:0	1.9	0.8	2.7	0.7	5.4	5.2	1.0	1.6
15:1 ω 8c	0.3	0.2	0.6	0.3	3.4	2.7	0.8	3.4
16:0i	4.4	2.3	0.5	0.3	1.0	1.4	1.8	2.5
16:0	20.2	33.0	22.3	40.6	9.3	16.2	10.0	9.4
16:1ω7c	44.1	43.0	46.3	35.4	25.6	30.3	60.7	54.2
17:0a	0.7	2.0	ND	0.3	0.3	0.7	ND	ND
17:0	1.4	2.1	1.6	1.8	4.4	6.0	0.7	1.3
17:1ω8c	6.2	3.2	8.1	2.8	18.2	13.6	4.6	5.0
17:1 ω 6c	0.2	ND	0.3	ND	1.4	1.4	0.4	0.6
18:0	0.4	1.9	0.2	2.2	0.4	1.1	0.4	0.4
18:1ω7c	12.6	5.2	11.6	10.1	7.3	7.8	16.3	12.6
19:1	ND	ND	ND	ND	1.4	1.5	ND	ND

241^T), respectively, while the relative amounts of C_{16:0} and C_{16:1 ω 7c} were constant or changed only slightly. *A. macleodii* did not show noticeable differences in the content of C_{17:1 ω 8c} and C_{18:1 ω 7c} when grown at different temperatures (Table 2).

The phospholipid compositions of strains KMM 241^T and KMM 642 were similar and included phosphatidylethanolamine (72.1 and 69.4 %, respectively), phosphatidylglycerol (23.2 and 23.1 %), diphosphatidylglycerol (2.5 and 3.4 %) and bis-phosphatidic acid (2.2 and 4.0 %).

The DNA G+C contents of KMM 241^T and KMM 642 were respectively 44.8 and 44.6 mol%. DNA–DNA hybridization experiments between the two strains showed DNA binding of 71 %.

16S rDNA sequence analysis revealed that the novel isolate KMM 241^T is a phylogenetic neighbour of *Glaciecola* sp. strain HA02, described as decomposing *Laminaria* thallus (99.7 % similarity; accession number AB049729). Strain KMM 241^T is phylogenetically closely related to *G. punicea* and *G. pallidula* (respectively 94.5 and 94.8 % sequence similarity) and somewhat less closely related to *A. macleodii* (93.4 % similarity). The 16S rRNA gene sequence similarity between *G. pallidula* and *G. punicea* was 93.3 %. Different algorithms consistently placed strain KMM 241^T adjacent to the two *Glaciecola* type strains and the neighbour-joining dendrogram is shown in Fig. 2. The branching is supported by bootstrap values above 75 %.

The phenotypic (Table 1) and chemotaxonomic (Table 2) characteristics of the novel isolates are consistent with

the description of *Glaciecola* species, including motility, requirement for sodium ions for growth, absence of growth in 10 % NaCl, the presence of oxidase, catalase and α -galactosidase activities, β -galactosidase production, the ability to hydrolyse aesculin, the lack of gelatinase, caseinase and urease activities, weak or delayed acid production from D-glucose, D-galactose and melibiose, C_{16:0}, C_{16:1 ω 7c} and C_{18:1 ω 7c} as major fatty acids and a G+C content of 44.6–44.8 mol%.

The novel bacteria can be distinguished from *Glaciecola* species by the following characteristics: the inability to produce pigments, to form filaments and to grow at 0–4 °C and the ability to degrade agar, carrageenan and DNA (weakly) and to utilize D-glucose, sucrose,

Fig. 2. Neighbour-joining analysis of 16S rRNA gene sequences showing the position of strains KMM 241^T among *Glaciecola* species. Percentages at branching points refer to bootstrap analyses (500 resamplings). Scale bar, 10 inferred nucleotide substitutions per 100 nucleotides.

D-mannitol, glycyl L-glutamic acid, cellobiose, maltose, D-galactose, D-fructose, D-mannose, L-serine, D-trehalose and β -hydroxybutyric acid.

On the basis of their phenotypic, chemotaxonomic, genomic and phylogenetic characteristics, we conclude that strains KMM 241^T and KMM 642 should be placed in the same species within the genus *Glaciecola* as *Glaciecola mesophila* sp. nov.

Description of *Glaciecola mesophila* sp. nov.

Glaciecola mesophila (me.so'phi.la. Gr. adj. *mesos* medium; Gr. adj. *philos* loving; N.L. fem. adj. *mesophila* medium-temperature-loving, mesophilic).

Aerobic, Gram-negative, oxidase- and catalase-positive, non-pigmented, non-spore-forming, ovoid or curved rod-shaped cells, 1.2–1.5 μ m long and 0.6–0.8 μ m in diameter, motile with single unsheathed polar flagella. Does not form filaments. On MA, forms smooth, convex, non-pigmented colonies, translucent or whitish with regular edges, 3–5 mm in diameter, depressed into the agar. Slightly halophilic. Requires sodium ions for growth (1–8% NaCl), optimum between 2 and 5%; no growth in 10% NaCl. Growth occurs between 7 and 35 °C, but not at 4–5 or 40 °C; optimum between 25 and 28 °C. The pH range is 5.5–9.5, optimum at pH 8.0–8.5. Chemo-organoheterotroph. Positive for lipase, amylase and β -galactosidase and α -galactosidase, decomposition of agar and carrageenan. Produces acid weakly from carbohydrates. Other phenotypic and biochemical tests are listed in Table 1. Predominant fatty acids are C_{16:0}, C_{16:1 ω 7c} and C_{18:1 ω 7c}. Phospholipids include phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and bis-phosphatidic acid. The G+C content of the DNA is 44.6–44.8 mol% (thermal denaturation method).

The type strain, strain KMM 241^T (=DSM 15026^T), was isolated from the ascidian *Halocynthia aurantium* in coastal sea water of the Sea of Japan. A reference strain, strain KMM 642, was isolated from a sponge *Plocamia* sp. at a depth of 350 m near the Komandorskie Islands.

Acknowledgements

We thank Ina Kramer and Jolantha Swiderski for excellent technical assistance in 16S rDNA sequencing and data analysis. We are grateful to Dr Susanne Verbarg and Anja Frühling for providing API and Biolog tests. This study was supported by grant no. 02-04-49517 of the Russian Foundation for Basic Research, grant 95-01/03-19 from the Ministry for Industry and Science (MIS) of the Russian Federation (RF) and a grant from the Programme

'Biodiversity' of the MIS RF and Russian Academy of Sciences.

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