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Mizugakiibacter sediminis gen. nov., sp. nov., isolated from a
freshwater lake

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Running head: *Mizugakiibacter sediminis* gen. nov., sp. nov.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of
strain skMP5^T is AB917594.

Summary

A novel moderately thermophilic bacterial strain skMP5^T was isolated from sediment of a freshwater lake in Japan. The cells were rod-shaped, motile, and Gram-negative. Growth was observed at temperatures ranging from 25 to 52°C, and the optimum growth was observed at 48–50°C. The range of pH for growth was 5.0–8.2, and the optimum pH was 6.0–7.0. The G+C content of genomic DNA was 72 mol%. The major components in the fatty acid profile were iso-C_{17:0}, iso-C_{17:1ω9c}. The predominant isoprenoid quinone of the strain was ubiquinone Q-8. The strain was facultatively anaerobic, and reduced nitrate to nitrite under anoxic conditions. Phylogenetic analysis based on 16S rRNA gene sequence indicated that the isolate was a member of the family *Xanthomonadaceae* within the class *Gammaproteobacteria*, and it showed highest sequence similarity with *Tahibacter aquaticus* RaM5-2 (93.6%) and *Metallibacterium scheffleri* DKE6^T (93.3%). On the basis of its phylogenetic and phenotypic properties, the strain skMP5^T (=DSM 27098^T = NBRC 109608^T) is proposed as the type strain of a new species of a novel genus, *Mizugakiibacter sediminis* gen. nov., sp. nov.

Lake Mizugaki is a freshwater lake in Japan, where some culture-independent studies were performed to investigate microbial community (Kojima *et al.*, 2009; Tsutsumi *et al.*, 2011; Kojima *et al.*, 2014). In recent years, several novel bacteria were isolated and described from water and sediment of this lake (Kojima & Fukui, 2010; Kojima & Fukui, 2011; Watanabe *et al.*, 2013; Watanabe *et al.*, 2014). In this study, a novel member of the family *Xanthomonadaceae* within the class *Gammaproteobacteria* isolated from the sediment of Lake Mizugaki is characterized.

The strain skMP5^T was isolated from sediment of Lake Mizugaki via enrichment culture of autotrophic sulfur-oxidizing bacteria. The enrichment culture was established and maintained at 45°C, as described previously (Watanabe *et al.*, 2014). The medium used for the enrichment was bicarbonate-buffered low-salt defined medium (Kojima & Fukui, 2011). The composition of the medium was as follows (l⁻¹): 0.2 g MgCl₂ · 6H₂O, 0.1 g CaCl₂ · 2H₂O, 0.1 g NH₄Cl, 0.1 g KH₂PO₄, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture solution, 1 ml vitamin B₁₂ solution, 1 ml thiamine solution, 30 ml NaHCO₃ solution, and 1.5 ml Na₂S₂O₃ solution. All stock solutions were prepared as described previously (Widdel & Bak, 1992).

For isolation and characterization of the strain, 10-fold-diluted tryptone soy broth

supplemented with glucose was used as basal medium. The medium (henceforth designated as TSG medium) was prepared by dissolving 3 g l⁻¹ tryptone soy broth (Difco) and 10 g l⁻¹ glucose in distilled water and pH was adjusted to 6.5 with HCl. The strain was obtained by repeated streaking on TSG medium solidified with 1.5% agar.

Purity of the isolate was checked by phase contrast light microscopy and sequencing of the 16S rRNA gene fragments amplified with several universal PCR primer pairs. The tests of Gram-stain, catalase activity, and oxidase activity were performed as described previously (Kojima & Fukui, 2011). The genomic G+C content of the DNA was determined with the HPLC methods as described previously (Katayama-Fujimura *et al.*, 1984).

The cultures were incubated at 45°C without shaking unless otherwise specified, and each experiment was performed in duplicate. Effects of salt concentrations (0, 1, 2, 3, 4 and 5% w/v NaCl) and temperature (10, 15, 18, 22, 25, 28, 32, 37, 40, 42, 45, 48, 50, 52, and 55°C) on growth of the strain were tested under aerobic conditions by using the TSG medium. The effect of pH on the growth was tested with modified TSG media bufferd with 20 mM of citrate (pH 4.5, 4.9, 5.4), MES (pH 5.0, 5.2, 5.4, 5.6, 5.7, 6.1, 6.2, 6.4, 6.6, 6.8, 7.0), PIPES (pH 6.4, 6.8, 7.1), MOPS (pH 6.6, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2), or Tricine (pH 7.7, 8.1, 8.2, 8.4, 8.7, 8.8, 9.0).

Anaerobic growth was tested in anoxic R2A broth (Daigo) prepared by bubbling with N₂ gas. Fermentative growth was tested in the medium without additional electron acceptor. Growth dependent on anaerobic respiration was tested in the medium supplemented with nitrate (10 mM) or poorly crystalline Fe(III) oxide (10 mM). The stock slurry of poorly crystalline Fe(III) oxide was prepared as described previously (Lovley, 2013). Changes in concentrations of nitrate and nitrite were determined with ion chromatography.

Tests with the API 20E, API 20NE and API ZYM (bioMérieux) were performed with 14 hours old cultures grown in R2A broth (Daigo), generally according to the manufacturer's instructions. Cells were harvested by centrifugation, and incubation was performed at 42°C. The strips of API 20E and API 20NE were read after 48 hour incubation, and API ZYM strip was read after 4 hour incubation.

Fatty acid profile of the isolate was analyzed for cells grown under two conditions, with TSG liquid medium and R2A agar plate (Daigo). The fatty acid analysis was performed by using the Sherlock Microbial Identification System (Version 6.0; database, TSBA40; MIDI). Isoprenoid quinines were extracted from cells grown in R2A broth with shaking, and analyzed with HPLC as described previously (Nishijima *et al.*, 1997). Analysis of polar lipids was carried out by the DSMZ Identification Service.

Fragment of 16S rRNA gene was amplified with the primer pair 27F and 1492R (Lane, 1991), and the resulting PCR product was directly sequenced. The obtained sequence was aligned with related sequences retrieved from the DDBJ/EMBL/GenBank databases using the program CLUSTAL_X (Thompson *et al.*, 1997). Phylogenetic trees were constructed with the program MEGA version 5.05 (Tamura *et al.*, 2011).

The cells of the obtained isolate skMP5^T were motile Gram-negative rods (1.5–8.0 µm long and 0.4–0.6 µm wide), occurring singly or in pairs (Fig. S1). Spore formation was not observed. On the agar-solidified TSG medium, the strain formed almost colorless transparent colonies after 2 days incubation at 45°C. Colonies on R2A agar were slightly yellowish and translucent. The diameter of the colonies was approximately 1.2 mm. The strain was catalase-negative and oxidase-positive. The G+C content of the genomic DNA of novel isolate was 72 mol%. As major respiratory quinone, only ubiquinone Q-8 was detected.

Components of the fatty acid profile of the strain cultivated under two different conditions are shown in Table 1. The most predominant fatty acid was iso-C_{17:0} in both cases, accounting for more than a third of total. The second most predominant component was iso-C_{17:1}ω9c, consistently. The other major fatty acids (>5% of total in

both) were iso-C_{15:0}, iso-C_{11:0} 3-OH, iso-C_{16:0}, and an unknown fatty acid (ECL 11.798).

Among these, contents of iso-C_{15:0} and iso-C_{16:0} differed markedly between cells cultured under different conditions. Polar lipids of strain skMP5^T included phosphatidylethanolamine, phosphatidylglycerol, and several unidentified phospholipids (Supplementary Fig. S2).

Growth of the strain was observed over a temperature range between 25°C and 52°C, and the optimum was 48–50°C. The range of pH for growth was 5.0–8.2, and the optimum pH was 6.0–7.0. No growth was observed in the medium containing 3% or higher NaCl, and 2% NaCl exhibited negative effect on growth.

Growth in the defined medium used for the enrichment was tested with various substrates, but no stable growth was observed under nitrate-reducing conditions. A modified version of the medium buffered with 20 mM MOPS/NaOH was used to test aerobic growth, but none of tested substrate supported growth in the defined medium.

By culturing in various modified media, it was revealed that growth of the strain was sustained only in media containing tryptone, which cannot be replaced with yeast extract or Casamino acids. The strain could grow on tryptone water (0.05% v/w) without any additional constituent, and carbon source utilization of the strain could not be assessed by growth in any defined media.

Under anoxic conditions, the strain did not grow in the R2A without additional electron acceptor. Anaerobic growth was observed in the medium supplemented with nitrate, along with nitrate consumption and nitrite production. Poorly crystalline Fe(III) did not serve as electron acceptor to support growth of the strain.

In the test of API ZYM, activities of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β -glucosidase (2-naphthyl- β -D-galactopyranosidase) were detected. Weak activity of valine arylamidase was also observed, and activities of the other enzymes were not detected. In the API 20NE tests, reduction of nitrates to nitrite and hydrolysis of gelatin were detected. Weak positive signals were observed for hydrolysis of aesculin and β -galactosidase (*p*-nitrophenyl- β -D-galactopyranosidase) activity. No enzymatic activities were detected in the API 20E except for the gelatin hydrolysis. No growth or acid production was observed in tests of substrate assimilations in the strips of API 20E and API 20NE. Glucose assimilation was also tested with API OF medium (bioMérieux), and no growth or acid production was observed after 48 hour incubation at 42°C.

In the 16S rRNA gene sequence analysis, characterized strains which showed highest sequence similarities to strain skMP5^T were *Tahibacter aquaticus* RaM5-2 (93.6%) and *Metallibacterium scheffleri* DKE6^T (93.3%), belonging to the family

Xanthomonadaceae within the class *Gammaproteobacteria*. Affiliation of the novel isolate to the family *Xanthomonadaceae* was confirmed with the maximum-likelihood phylogenetic tree (Fig. 1), in which the novel strain formed a cluster with species of the genera *Metallibacterium*, *Dyella*, *Fulvimonas* and *Rhodanobacter*. This cluster was consistently observed in the trees constructed with the methods of minimum-evolution (Fig. S3) and neighbor-joining (data not shown). These analyses also indicated that none of the existing genera can accommodate the novel strain without loss of monophyleticity (Fig. 1, Fig. S3). In addition to the independent phylogenetic position and low sequence similarities (<94%) to the relatives, physiological properties of the novel strain were distinct from strains of the genera in this lineage (Table 2). On the basis of these phylogenetic and phenotypic properties, the strain skMP5^T is proposed to be assigned to a new species of a novel genus, with the name *Mizugakiibacter sediminis* gen. nov., sp. nov.

Description of *Mizugakiibacter* gen. nov.

Mizugakiibacter (Mi.zu.ga.ki.i.bac'ter. N.L. masc. n. bacter, a rod; N.L. masc. n. Mizugakiibacter, a rod isolated from Lake Mizugaki).

The G+C content of genomic DNA is around 72 mol%. Cells are Gram-negative,

catalase-negative and oxidase-positive. Based on 16S rRNA gene sequence analysis, phylogenetically affiliated to the family *Xanthomonadaceae* within the class *Gammaproteobacteria*. Major fatty acids are iso-C_{17:0} and iso-C_{17:1}ω9c. The predominant quinone is Q-8. The type species is *Mizugakiibacter sediminis*.

Description of *Mizugakiibacter sediminis* sp. nov.

Mizugakiibacter sediminis (se.di'mi.nis. L. gen. n. sediminis of sediment).

Cells are rod-shaped, 1.5–8.0 μm in length and 0.4–0.6 μm in width. Colonies on R2A agar are round, slightly yellowish and translucent. Growth occurs at temperatures between 25 and 55°C, with optimum growth at 48–50°C. The pH range for growth is 5.0–8.2, and optimum growth occurs at pH 6.0–7.0. The G+C content of genomic DNA is 72 mol%. Anaerobic growth occurs under the presence of nitrate. Reduces nitrate to nitrite and hydrolyzes gelatin. In the test of API ZYM, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-glucosidase (2-naphthyl-β-D-galactopyranosidase), and weakly positive for valine arylamidase. The type strain skMP5^T (=DSM 27098^T = NBRC 109608^T) was isolated from sediment of a freshwater lake in Japan.

179

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241

Table 1. Cellular fatty acid contents (percentage of total) of the strain skMP5^T, grown with TSG medium and R2A agar.

Fatty acid	TSG	R2A agar
C _{11:0} iso	1.44	0.69
C _{11:0} iso 3OH	8.43	8.89
C _{13:0} iso	0.28	-
C _{14:0} iso	0.25	-
C _{14:0}	0.25	-
C _{15:0} iso	17.47	9.93
C _{15:0} anteioso	0.9	0.66
C _{16:0} N alcohol	0.27	-
C _{16:0} iso	5.44	16.88
C _{16:0}	2.91	2.36
C _{17:1ω9c} iso	19.79	16.97
C _{17:0} iso	34.8	33.69
C _{17:0} anteioso	1.33	1.85
C _{18:0} iso	0.44	2.81
Summed feature 3	0.72	-
Unknown, ECL 11.798	5.27	5.27

Table 2. Differential physiological properties of skMP5^T and strains representing the related genera. Strains: 1, skMP5^T; 2, *Metallibacterium scheffleri* DKE6^T (Ziegler *et al.*, 2013); 3, *Dyella japonica* XD53^T (Xie & Yokota, 2005); 4, *Fulvimonas soli* LMG 19981^T (Mergaert *et al.*, 2002); 5, *Aquimonas voraii* GPTSA 20^T (Saha *et al.*, 2005). +, Positive; -, negative; W, weakly positive; VW, very weakly positive; NR, not reported.

Characteristics	1	2	3	4	5
Optimum temperature for growth (°C)	48–50	25–30	25–30	NR	NR
Growth at 20°C	-	+	+	-	-
Growth at 45°C	+	-	-	-	-
pH for growth					
Range	5.0–8.2	2–6.5	5.6–8.0	NR [*]	6.0–11.0
Optimum	6.0–7.0	5.5	6.5–7.2	NR	NR
DNA G+C content (%)	72	66.6	63.4	71.9	75
Oxidase	+	W	-	+	+
Catalase	-	VW	+	+	+
Motility	+	-	+	+	+
Nitrate reduction	+	-	+	-	-
Hydrolysis/liquefaction of gelatin	+	NR	-	-	+
Predominant fatty acids (>20%)	iso-C _{17:0}	iso-C _{17:0} iso-C _{17:1ω9c}	iso-C _{17:0} iso-C _{17:1ω9c} iso-C _{15:0}	iso-C _{15:0}	iso-C _{15:0}

* Enrichment, isolation and characterization were performed at pH 6.8–7.0.

256 Figure legends

257

258 Fig. 1 Maximum-likelihood tree showing the phylogenetic position of skMP5^T within
259 the family *Xanthomonadaceae*, based on the 16S rRNA gene sequence analysis.

260 *Legionella pneumophila* is included as an outgroup. Numbers on nodes represent

261 percentage values of 1000 bootstrap resampling (values greater than 50 are shown).