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

Hisaya Kojima*, Riho Tokizawa and Manabu Fukui

The Institute of Low Temperature Science, Hokkaido University. Kita-19, Nishi-8, Kita-ku, Sapporo 060-0819, Japan

*Corresponding author.

E-mail: kojimah@pop.lowtem.hokudai.ac.jp

Phone: +81-11-706-5460

Fax: +81-11-706-5460

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

Subject category: New taxa: *Proteobacteria*

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-$C_{17:1}\omega9c$. 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 $MgCl_2 . 6H_2O$, 0.1 g $CaCl_2 . 2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture solution, 1 ml vitamin B_{12} solution, 1 ml thiamine solution, 30 ml $NaHCO_3$ solution, and 1.5 ml $Na_2S_2O_3$ 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

53 supplemented with glucose was used as basal medium. The medium (henceforth
54 designated as TSG medium) was prepared by dissolving 3 g l^{-1} tryptone soy broth
55 (Difco) and 10 g l^{-1} glucose in distilled water and pH was adjusted to 6.5 with HCl. The
56 strain was obtained by repeated streaking on TSG medium solidified with 1.5% agar.
57 Purity of the isolate was checked by phase contrast light microscopy and sequencing of
58 the 16S rRNA gene fragments amplified with several universal PCR primer pairs. The
59 tests of Gram-stain, catalase activity, and oxidase activity were performed as described
60 previously (Kojima & Fukui, 2011). The genomic G+C content of the DNA was
61 determined with the HPLC methods as descried previously (Katayama-Fujimura *et al*.,
62 1984).
63 The cultures were incubated at 45°C without shaking unless otherwise specified, and
64 each experiment was performed in duplicate. Effects of salt concentrations (0, 1, 2, 3, 4
65 and 5% w/v NaCl) and temperature (10, 15, 18, 22, 25, 28, 32, 37, 40, 42, 45, 48, 50, 52,
66 and 55°C) on growth of the strain were tested under aerobic conditions by using the
67 TSG medium. The effect of pH on the growth was tested with modified TSG media
68 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,
69 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,
70 8.0, 8.2), or Tricine (pH 7.7, 8.1, 8.2, 8.4, 8.7, 8.8, 9.0).

71 Anaerobic growth was tested in anoxic R2A broth (Daigo) prepared by bubbling with
72 N_2 gas. Fermentative growth was tested in the medium without additional electron
73 acceptor. Growth dependent on anaerobic respiration was tested in the medium
74 supplemented with nitrate (10 mM) or poorly crystalline Fe(III) oxide (10 mM). The
75 stock slurry of poorly crystalline Fe(III) oxide was prepared as descried previously
76 (Lovley, 2013). Changes in concentrations of nitrate and nitrite were determined with
77 ion chromatography.
78 Tests with the API 20E, API 20NE and API ZYM (bioMérieux) were performed with
79 14 hours old cultures grown in R2A broth (Daigo), generally according to the
80 manufacturer's instructions. Cells were harvested by centrifugation, and incubation was
81 performed at 42°C. The strips of API 20E and API 20NE were read after 48 hour
82 incubation, and API ZYM strip was read after 4 hour incubation.
83 Fatty acid profile of the isolate was analyzed for cells grown under two conditions,
84 with TSG liquid medium and R2A agar plate (Daigo). The fatty acid analysis was
85 performed by using the Sherlock Microbial Identification System (Version 6.0; database,
86 TSBA40; MIDI). Isoprenoid quinines were extracted from cells grown in R2A broth
87 with shaking, and analyzed with HPLC as described previously (Nishijima *et al*., 1997).
88 Analysis of polar lipids was carried out by the DSMZ Identification Service.

89 Fragment of 16S rRNA gene was amplified with the primer pair 27F and 1492R (Lane,
90 1991), and the resulting PCR product was directly sequenced. The obtained sequence
91 was aligned with related sequences retrieved from the DDBJ/EMBL/GenBank databases
92 using the program CLUSTAL_X (Thompson *et al*., 1997). Phylogenetic trees were
93 constructed with the program MEGA version 5.05 (Tamura *et al*., 2011).
94
95 The cells of the obtained isolate skMP5^T were motile Gram-negative rods (1.5–8.0
96 μm long and 0.4–0.6 μm wide), occurring singly or in pairs (Fig. S1). Spore formation
97 was not observed. On the agar-solidified TSG medium, the strain formed almost
98 colorless transparent colonies after 2 days incubation at 45°C. Colonies on R2A agar
99 were slightly yellowish and translucent. The diameter of the colonies was approximately
100 1.2 mm. The strain was catalase-negative and oxidase-positive. The G+C content of the
101 genomic DNA of novel isolate was 72 mol%. As major respiratory quinine, only
102 ubiquinone Q-8 was detected.
103 Components of the fatty acid profile of the strain cultivated under two different
104 conditions are shown in Table 1. The most predominant fatty acid was iso-$C_{17:0}$ in both
105 cases, accounting for more than a third of total. The second most predominant
106 component was iso-$C_{17:1}\omega9c$, 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 reveled 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.

125 Under anoxic conditions, the strain did not grow in the R2A without additional
126 electron acceptor. Anaerobic growth was observed in the medium supplemented with
127 nitrate, along with nitrate consumption and nitrite production. Poorly crystalline Fe(III)
128 did not serve as electron acceptor to support growth of the strain.
129 In the test of API ZYM, activities of alkaline phosphatase, esterase (C4), esterase
130 lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase,
131 and β-glucosidase (2-naphthyl-β-D-galactopyranosidase) were detected. Weak activity
132 of valine arylamidase was also observed, and activities of the other enzymes were not
133 detected. In the API 20NE tests, reduction of nitrates to nitrite and hydrolysis of gelatin
134 were detected. Weak positive signals were observed for hydrolysis of aesculin and
135 β-galactosidase (*p*-nitrophenyl-β-D-galactopyranosidase) activity. No enzymatic
136 activities were detected in the API 20E except for the gelatin hydrolysis. No growth or
137 acid production was observed in tests of substrate assimilations in the strips of API 20E
138 and API 20NE. Glucose assimilation was also tested with API OF medium (bioMérieux),
139 and no growth or acid production was observed after 48 hour incubation at 42°C.
140 In the 16S rRNA gene sequence analysis, characterized strains which showed highest
141 sequence similarities to strain skMP5^T were *Tahibacter aquaticus* RaM5-2 (93.6%) and
142 *Metallibacterium scheffleri* DKE6^T (93.3%), belonging to the family

143 *Xanthomonadaceae* within the class *Gammaproteobacteria*. Affiliation of the novel
144 isolate to the family *Xanthomonadaceae* was confirmed with the maximum-likelihood
145 phylogenetic tree (Fig. 1), in which the novel strain formed a cluster with species of the
146 genera *Metallibacterium*, *Dyella*, *Fulvimonas* and *Rhodanobacter*. This cluster was
147 consistently observed in the trees constructed with the methods of minimum-evolution
148 (Fig. S3) and neighbor-joining (data not shown). These analyses also indicated that none
149 of the existing genera can accommodate the novel strain without loss of
150 monophyleticity (Fig. 1, Fig. S3). In addition to the independent phylogenetic position
151 and low sequence similarities (<94%) to the relatives, physiological properties of the
152 novel strain were distinct from strains of the genera in this lineage (Table 2). On the
153 basis of these phylogenetic and phenotypic properties, the strain skMP5^T is proposed to
154 be assigned to a new species of a novel genus, with the name *Mizugakiibacter sediminis*
155 gen. nov., sp. nov.
156
157 Description of *Mizugakiibacter* gen. nov.
158 *Mizugakiibacter* (Mi.zu.ga.ki.i.bac'ter. N.L. masc. n. bacter, a rod; N.L. masc. n.
159 Mizugakiibacter, a rod isolated from Lake Mizugaki).
160 The G+C content of genomic DNA is around 72 mol%. Cells are Gram-negative,

161 catalase-negative and oxidase-positive. Based on 16S rRNA gene sequence analysis,
162 phylogenetically affiliated to the family *Xanthomonadaceae* within the class
163 *Gammaproteobacteria*. Major fatty acids are iso-$C_{17:0}$ and iso-$C_{17:1}\omega9c$. The
164 predominant quinone is Q-8. The type species is *Mizugakiibacter sediminis*.
165
166 Description of *Mizugakiibacter sediminis* sp. nov.
167 *Mizugakiibacter sediminis* (se.di'mi.nis. L. gen. n. sediminis of sediment).
168 Cells are rod-shaped, 1.5–8.0 μm in length and 0.4–0.6 μm in width. Colonies on R2A
169 agar are round, slightly yellowish and translucent. Growth occurs at temperatures
170 between 25 and 55°C, with optimum growth at 48–50°C. The pH range for growth is
171 5.0–8.2, and optimum growth occurs at pH 6.0–7.0. The G+C content of genomic DNA
172 is 72 mol%. Anaerobic growth occurs under the presence of nitrate. Reduces nitrate to
173 nitrite and hydrolyzes gelatin. In the test of API ZYM, positive for alkaline phosphatase,
174 esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase,
175 naphthol-AS-BI-phosphohydrolase, and β-glucosidase
176 (2-naphthyl-β-D-galactopyranosidase), and weakly positive for valine arylamidase. The
177 type strain skMP5^T (=DSM 27098^T = NBRC 109608^T) was isolated from sediment of a
178 freshwater lake in Japan.

ACKNOWLEDGMENTS

We thank T. Iwata and Daimon and Shiokawa Dams Management Office, for their support in sampling of lake sediment. This study was supported by a grant from the Institute for fermentation, Osaka, to Kojima.

REFERENCES

Katayama-Fujimura, Y., Komatsu, Y., Kuraishi, H. & Kaneko, T. (1984). Estimation of DNA base composition by high-performance liquid chromatography of its Nuclease PI hydrolysate. *Agric Bio Chem* **48**, 3169–3172.

Kojima, H. & Fukui, M. (2010). *Sulfuricella denitrificans* gen. nov., sp. nov., a sulfur-oxidizing autotroph isolated from a freshwater lake. *Int J Syst Evol Microbiol* **60**, 2862–2866.

Kojima, H. & Fukui, M. (2011). *Sulfuritalea hydrogenivorans* gen. nov., sp. nov., a facultative autotroph isolated from a freshwater lake *Int J Syst Evol Microbiol* **61,** 1651–1655.

Kojima, H., Iwata, T. & Fukui, M. (2009). DNA-based analysis of planktonic methanotrophs in a stratified lake. *Freshw Biol* **54,** 1501–1509.

Kojima, H., Watanabe, T., Iwata, T. & Fukui, M. (2014). Identification of major planktonic sulfur oxidizers in stratified freshwater lake. *Plos One* **9,** e93877.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, chapter 6, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.

Lovley, D. R. (2013). Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. In The Prokaryotes, 4th edn, pp. 287–308. Edited by Rosenberg E., DeLong E. F., Lory S., Stackebrandt E., Thompson F. Berlin: Springer.

Mergaert, J., Cnockaert, M. C. & Swings, J. (2002). *Fulvimonas soli* gen. nov., sp. nov., a gamma-proteobacterium isolated from soil after enrichment on acetylated starch plastic. *Int J Syst Evol Microbiol* **52,** 1285–1289.

Nishijima, M., Araki-Sakai, M. & Sano, H. (1997). Identification of isoprenoid quinones by frit-FAB liquid chromatography-mass spectrometry for the chemotaxonomy of microorganisms. *J Microbiol Methods* **28,** 113–122.

Saha, P., Krishnamurthi, S., Mayilraj, S., Prasad, G.S., Bora, T.C. & Chakrabarti, T. (2005). *Aquimonas voraii* gen. nov., sp. nov., a novel gammaproteobacterium isolated from a warm spring of Assam, India. *Int J Syst Evol Microbiol* **55,** 1491–1495.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011).

MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Tsutsumi, M., Iwata, T., Kojima, H. & Fukui, M. (2011) Spatiotemporal variations in an assemblage of closely related planktonic aerobic methanotrophs. *Freshw Biol* **56,** 342–351.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25,** 4876–4882.

Watanabe, M., Kojima, H. & Fukui, M. (2013). *Desulfotomaculum intricatum* sp. nov., a sulfate reducer isolated from freshwater lake sediment. *Int J Syst Evol Microbiol* **63,** 3574–3578.

Watanabe, M., Kojima, H. & Fukui, M. (2014). Proposal of *Effusibacillus lacus* gen. nov. sp. nov., and reclassification of *Alicyclobacillus pohliae* as *Effusibacillus pohliae* comb. nov. and *Alicyclobacillus consociatus* as *Effusibacillus consociatus* comb. nov. *Int J Syst Evol Microbiol* **64,** 2770–2774.

Widdel, F. & Bak, F. (1992). Gram-negative mesotrophic sulfate-reducing bacteria. In *The Prokaryotes* 2nd edn, vol. 4, pp. 3352–3378. Edited by A. Balows, H. G. Tru¨per, M.

Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.

Xie, C.-H. & Yokota, A. (2005). *Dyella japonica* gen. nov., sp. nov., a cproteobacterium isolated from soil. *Int J Syst Evol Microbiol* **55,** 753–756.

Ziegler, S., Waidner, B., Itoh, T., Schumann, P., Spring, S. & Gescher J. (2013). *Metallibacterium scheffleri* gen. nov., sp. nov., an alkalinizing gammaproteobacterium isolated from an acidic biofilm. *Int J Syst Evol Microbiol* **63,** 1499–1504.

242

243 Table 1. Cellular fatty acid contents (percentage of total) of the strain skMP5^{T}, grown

244 with TSG medium and R2A agar.

245

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_{17:1}\omega$9c 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

246

247

248 Table 2. Differential physiological properties of skMP5^T and strains representing the

249 related genera. Strains: 1, skMP5^T; 2, *Metallibacterium scheffleri* DKE6^T (Ziegler *et al*.,

250 2013); 3, *Dyella japonica* XD53^T (Xie & Yokota, 2005); 4, *Fulvimonas soli* LMG

251 19981^T (Mergaert *et al*., 2002); 5, *Aquimonas voraii* GPTSA 20^T (Saha *et al*., 2005). +,

252 Positive; -, negative; W, weakly positive; VW, very weakly positive; NR, not reported.

253

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-$C_{17:1}\omega9c$	iso-$C_{17:0}$ iso-$C_{17:1}\omega9c$ iso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$

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

255

256 Figure legends

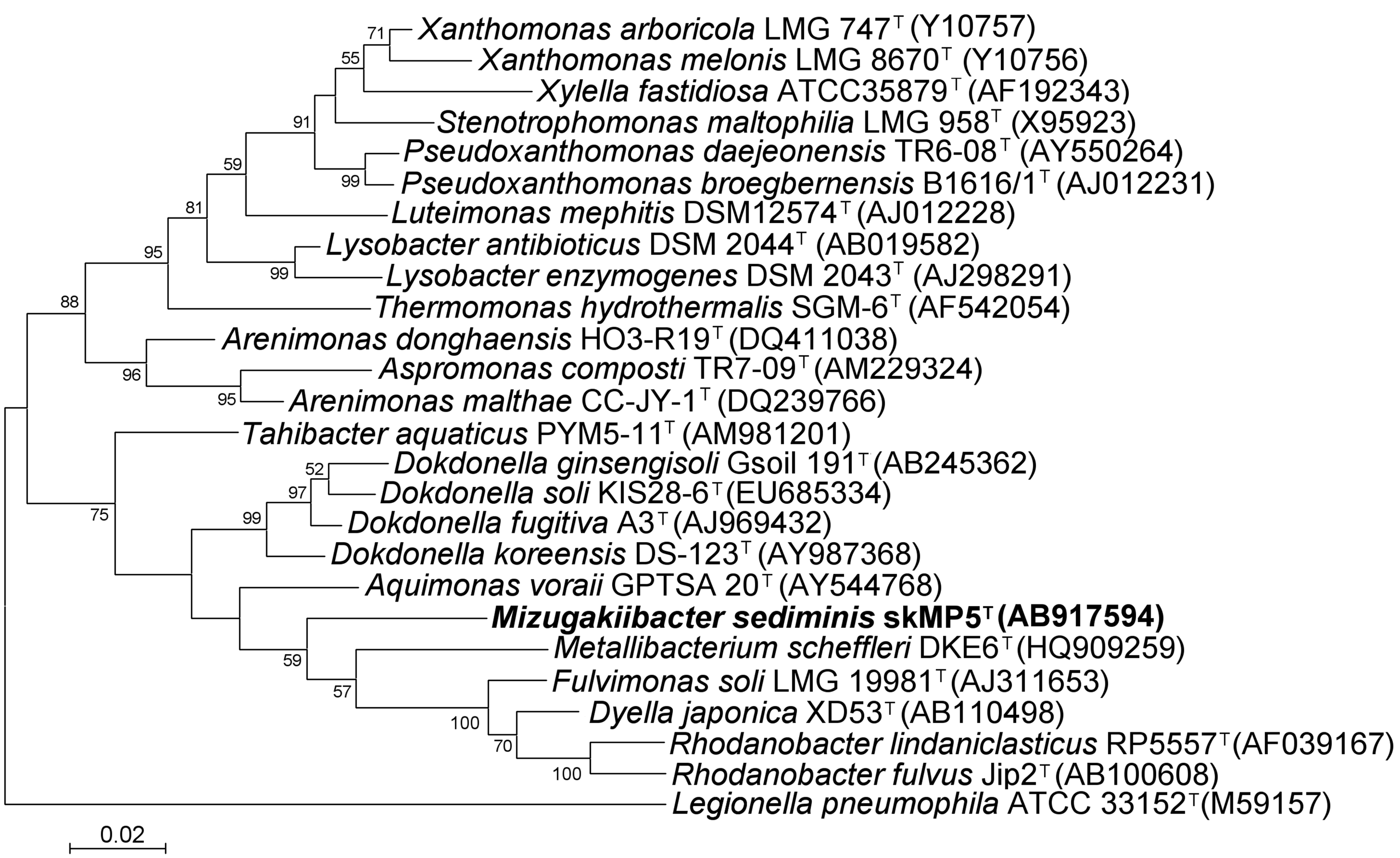
257

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).


Xanthomonas arboricola LMG 747T (Y10757)
Xanthomonas melonis LMG 8670T (Y10756)
Xylella fastidiosa ATCC35879T (AF192343)
Stenotrophomonas maltophilia LMG 958T (X95923)
Pseudoxanthomonas daejeonensis TR6-08T (AY550264)
Pseudoxanthomonas broegbernensis B1616/1T (AJ012231)
Luteimonas mephitis DSM12574T (AJ012228)
Lysobacter antibioticus DSM 2044T (AB019582)
Lysobacter enzymogenes DSM 2043T (AJ298291)
Thermomonas hydrothermalis SGM-6T (AF542054)
Arenimonas donghaensis HO3-R19T (DQ411038)
Aspromonas composti TR7-09T (AM229324)
Arenimonas malthae CC-JY-1T (DQ239766)
Tahibacter aquaticus PYM5-11T (AM981201)
Dokdonella ginsengisoli Gsoil 191T (AB245362)
Dokdonella soli KIS28-6T (EU685334)
Dokdonella fugitiva A3T (AJ969432)
Dokdonella koreensis DS-123T (AY987368)
Aquimonas voraii GPTSA 20T (AY544768)
Mizugakiibacter sediminis skMP5T (AB917594)
Metallibacterium scheffleri DKE6T (HQ909259)
Fulvimonas soli LMG 19981T (AJ311653)
Dyella japonica XD53T (AB110498)
Rhodanobacter lindaniclasticus RP5557T (AF039167)
Rhodanobacter fulvus Jip2T (AB100608)
Legionella pneumophila ATCC 33152T (M59157)
71
55
91
59
99
81
95
99
88
96
95
52
97
75
99
59
57
100
70
100
0.02