# *Acidocella aquatica* sp. nov., a novel acidophilic heterotrophic bacterium isolated from a freshwater lake

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain Ok2G$^T$ is LC199502.

**Summary**

A novel acidophilic heterotrophic bacterium, strain Ok2G$^{T}$, was isolated from a freshwater lake in Japan. Cells of the isolate were Gram-stain-negative and non-motile rods (0.6–0.8×1.0–2.8 μm). Growth was observed at 4–35°C with an optimum growth temperature of 28°C. The range of pH for growth was 3.0–6.2 with an optimum pH of 4.5. The strain utilized fructose, glucose, sucrose, mannitol, sorbitol, ethanol, benzyl alcohol, pyruvate, yeast extract and tryptone as carbon and energy sources for aerobic growth. DNA G+C content was 62.6 mol%. The major cellular fatty acid and isoprenoid quinone were summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and Q-10, respectively. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain Ok2G$^{T}$ belongs to the genus *Acidocella* but is distinct from existing species with sequence similarities lower than 97%. On the basis of these results, strain Ok2G$^{T}$ (=NBRC 112502$^{T}$ =DSM 104037$^{T}$) is proposed as the type strain of a novel species, *Acidocella aquatica* sp. nov.

The genus *Acidocella* within the class *Alphaproteobacteria* was proposed in 1995, along with an emendation of the genus *Acidiphilium* [1]. It originally contained two species transferred from the genus *Acidiphilium* (*A. facilis* and *A. aminolytica*), and *A. aluminiidurans* was added to the genus later [2]. In addition to these three species with validly published name, "*A. aromatica*" was also proposed in this genus [3]. All type strains of these species are acidophilic and mesophilic heterotrophs isolated from acidic environments [2, 3, 4, 5]. Some other members of this genus have also been isolated from environments affected by acidic mine drainage [6, 7, 8]. In this study, a novel bacterium related to these organisms was isolated, and its physiological, biochemical and phylogenetic characteristics are reported.

Strain Ok2G$^T$ was isolated from a freshwater lake (Lake Okotanpe) in Hokkaido, northeastern Japan. A sample of lake water was taken at 10 m depth from the lake surface. Temperature and pH of the sample was 11.3°C and 5.9 respectively. An aliquot of the sample (0.5 ml) was inoculated into 10 ml of a synthetic liquid medium consisting of the following constituents ($l^{-1}$): 0.5 g glucose, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.1 g KCl, 0.2 g $NaNO_3$, 0.1 g methylphosphonic acid and 1 ml trace element solution SL-10 [9]. The culture was incubated under aerobic conditions in the dark at 15°C. A pure culture of strain Ok2G$^T$ was obtained by repeated serial dilution in the same medium. The culture purity was checked by microscopy, observation of colony morphology and repeated sequencing of the 16S rRNA gene fragments amplified with universal PCR primer pairs.

For physiological and biochemical characterization of strain Ok2G$^T$, another synthetic medium was prepared. The medium was modified from that used for isolation, to contain $NH_4Cl$ (0.1 g $l^{-1}$) and $KH_2PO_4$ (0.1 g $l^{-1}$), instead of $NaNO_3$ and methylphosphonic acid. The concentration of glucose was increased to 1 g $l^{-1}$ and pH was adjusted to 3–5 with hydrochloric acid. This basal medium was used for the characterization unless otherwise specified.

Cell morphology was examined by phase-contrast microscopy (Axioplan 2; Zeiss). Colony morphology was observed on 1.5% (w/v) agar-solidified basal medium with increased glucose concentration of 10 g $l^{-1}$. The

Gram-staining test was carried out using a kit (Fluka). Catalase activity was assessed by pouring 3% $H_2O_2$ solution onto a pellet of cells. Oxidase activity was carried out using an oxidase test reagent (bioMérieux). The genomic G+C content of the DNA was determined with HPLC methods [10], using a kit (Yamasa Shoyu). Isoprenoid quinone was analyzed at Techno Suruga (Shizuoka, Japan) by using the ACQUITY UPLC system (Waters).

The cellular fatty acid profile of strain Ok2G$^T$ was analyzed in parallel with those of the type strains representing *Acidocella* species as references for comparisons. The following strains were purchased from DSM, ATCC and NBRC; "*A. aromatica*" DSM 27026 (=PFBC), *A. aminolytica* DSM 11237 (=101$^T$), *A. aluminiidurans* NBRC 104303 (=AL46$^T$) and *A. facilis* ATCC 35904 (=PW2$^T$). All strains were cultured under identical conditions using the basal medium containing fructose (1 g $l^{-1}$), instead of glucose. The strains were cultured at 28°C for 5 days without shaking, and cells at late log-phase were harvested by centrifugation. The fatty acid analysis was performed by using the Sherlock Microbial Identification System Version 6.0, with database of TSBA6 (MIDI).

To determine the effects of temperature and pH on growth of strain Ok2G$^T$, cells were cultured in the basal medium supplemented with 0.05% (w/v) yeast extract, and its growth was monitored as optical density measured at 660 nm. The effects of temperature were examined by culturing the strain at 0, 4, 8, 13, 15, 18, 22, 25, 28, 32, 35, 37 and 45°C. The effects of pH were examined at 15°C by culturing the strains on the medium with pH initial of 1.6, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.2, 6.4 and 6.6. Growth inhibition by 0.25 mM acetate was tested at pH 3.6 as described previously [11].

Utilization of organic substrates for growth was tested at 25°C with the basal medium without glucose, supplemented with one of the following substrates (numbers without units indicate concentration in mM) as carbon and energy sources: L-arabinose (5), D-fructose (5), D-galactose (5), D-glucose (5), D-mannose (5), D-xylose (5), D-cellobiose (5), D-sucrose (5), glycerol (10), D-mannitol (5), sorbitol (5), ethanol (15), benzyl alcohol (5), methanol (20), phenol (5), sodium acetate (10), sodium benzoate (1), sodium citrate (5), sodium lactate (10), sodium propionate (10), sodium pyruvate (10), tryptone (0.04 %) and yeast extract (0.04 %).

Under identical conditions, substrate utilizations of the reference strains mentioned above were also tested for direct comparisons.

For phylogenetic analysis, the 16S rRNA gene was amplified by PCR using the primer pair 27F and 1492R [12] and then directly sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The primers used for sequencing are listed in Table S1. The resulting sequence of strain Ok2G$^T$ was aligned together with the sequences of the related species, by using the ClustalX version 2.1 [13]. All positions with gaps were excluded excluded for further analysis, and 1244 positions were included in the final dataset. The evolutionary distances were computed with Tamura 3-parameter model, using the program *MEGA* version 6.0 [14]. Phylogenetic trees were reconstructed with the neighbor-joining and minimum-evolution methods. Bootstrap analysis was performed for 500 replicates.

Cells of strain Ok2G$^T$ grown at 15°C were non-motile and non-endospore forming rods (1.0–2.8 μm long and 0.6–0.8 μm wide) (Fig. S1). On the solid medium, strain Ok2G$^T$ formed domed, round and beige colonies. The cells were weakly positive for catalase and oxidase negative. Gram-staining was negative. DNA G+C content was 62.6 mol%. The major isoprenoid quinone was Q-10.

Strain Ok2G$^T$ grew at temperatures between 4 and 35°C, with optimum temperature of 28°C (Table 1). The pH range for growth was pH 3.0 to 6.2, with optimum pH of pH 4.5. As carbon and energy sources to support growth, strain Ok2G$^T$ utilized some sugars and alcohols, as summarized in Table 1 and in the description of the species. The substrate utilizations of the other strains tested in the same medium are also shown in Table 1.

As shown in Table 2, the most predominant cellular fatty acid of strain Ok2G$^T$ grown on fructose at 28°C was summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), accounting for 73.4% of the total. The summed feature 8 was also dominant in the fatty acid profiles of all strains tested, but considerable differences in the compositions were observed among the strains (Table 2). The cellular fatty acid profile of strain Ok2G$^T$ was characterized by relatively high content of $C_{16:0}$ 3-OH, in comparison to the other strains cultured under the

same conditions. On the other hand, $C_{16:0}$ 2-OH and summed feature 2 ($C_{14:0}$ 3-OH and/or iso $C_{16:1}$ I) were not detected in strain Ok2G$^T$ but detected in the other strains.

Among the type strains of species with valid published name, *A. aluminiidurans* AL46$^T$ showed the highest 16S rRNA gene similarity (96.5 %) to strain Ok2G$^T$, followed by *A. facilis* PW2$^T$ (96.3 %) and with *A. aminolytica* 101$^T$ (95.5 %). In the phylogenetic analysis including these strains, an identical tree topology was obtained by the methods of minimum-evolution and neighbor-joining (Fig. 1). The tree topology indicated that strain Ok2G$^T$ cannot be assigned to any existing species in the genus *Acidocella* (Fig .1). The analysis also indicated that strain Ok2G$^T$ form a robust cluster with strains isolated from acidic mine drainage-impacted sites in geographically distant localities [6, 7, 8]. This cluster was formed by the strains sharing high sequence similarity (>98%), and may represent a novel species of the genus *Acidocella*.

Some differential properties between Ok2G$^T$ and the type strains of *Acidocella* species are shown in Table 1. As a unique feature, strain Ok2G$^T$ can grow at temperatures below the lower limits of the other species. Some differences were also observed in the utilization of substrates for growth (Table 1). In addition, the cellular fatty acid profile of strain Ok2G$^T$ was distinct from those of the strains representing existing species culture under the same conditions (Table 2). In conclusion, strain Ok2G$^T$ should represent a novel species of the genus *Acidocella*, and *Acidocella aquatica* sp. nov. is proposed with the type strain Ok2G$^T$ (= NBRC 112502$^T$ = DSM 104037$^T$).

**Description of *Acidocella aquatica* sp. nov.**

*Acidocella aquatica* (a.qua′ti.ca. L. fem. adj. *aquatica* aquatic; from freshwater). Aerobic, Gram-stain-negative, non-spore forming and non-motile rods, 1.0–2.8 µm long and 0.6–0.8 µm wide. Growth occurs at 4–35°C with optimum temperature of 28°C and at pH 3.0–6.2 with optimum pH of 4.5. The cells are weakly positive for catalase and oxidase negative. Grows heterotrophically with fructose, glucose, sucrose, mannitol, sorbitol, ethanol, benzyl alcohol, pyruvate, tryptone and yeast extract. Arabinose, galactose, mannose, xylose, cellobiose, glycerol, methanol, phenol, sodium acetate, sodium benzoate, sodium citrate,

sodium lactate, and sodium propionate do not support the growth. DNA G+C content of the type strain is 62.6 mol%. Major cellular fatty acids and isoprenoid quinone are summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) and Q-10, respectively. The type strain Ok2G$^T$ (=NBRC 112502$^T$ =DSM 104037$^T$) was isolated from a freshwater lake (Lake Okotanpe) in Japan.


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## Conflicts of interest statement

The authors declare that there is no conflict of interest.

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Table 1. Differential characteristics of strain Ok2G$^T$ and type strains of *Acidocella* species. Strain: 1, strain Ok2G$^T$ (this study); 2, *A. aluminiidurans* AL46$^T$ [2]; 3, *A. facilis* PW2$^T$ [1, 4]; 4, *A. aminolytica* 101$^T$ [5]; 5, "*A. aromatica*" PFBC [3]. Growth substrates were tested for all strains in this study. The other data were taken from respective references. +, positive; -, negative; NR, not reported. All strains grew on fructose, sucrose, and benzyl alcohol. Under the tested conditions, all strains did not grow on mannose, cellobiose, methanol, acetate, citrate, propionate or phenol.

| Strain | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Motility | - | - | + | + | + |
| Temperature range (°C) | 4–35 | 17–42 | nr | 25–37 | 15–37.5 |
| DNA G+C content (mol %) | 62.6 | 65.6 | 64.4 | 58.7 | 61.6-63.9 |
| Growth on | | | | | |
| Arabinose | - | + | + | + | - |
| Galactose | - | + | + | + | - |
| Glucose | + | + | + | + | - |
| Xylose | - | - | + | + | - |
| Glycerol | - | + | + | - | - |
| Mannitol | + | + | + | + | - |
| Sorbitol | + | - | - | + | + |
| Ethanol | + | + | + | + | - |
| Benzoate | - | + | - | + | + |
| Lactate | - | + | + | + | - |
| Pyruvate | + | + | + | + | - |
| Tryptone | + | + | + | + | - |
| Yeast extract | + | + | + | + | - |

Table 2. Cellular fatty acid contents (% of total) of strain Ok2G$^T$ and type strains of *Acidocella* species grown on fructose at 28°C. Fatty acids less than 1% in all strains are not shown. Strains: 1, Ok2G$^T$; 2, *A. aluminiidurans* AL46$^T$; 3, *A. facilis* PW2$^T$; 4, *A. aminolytica*; 5, "*A. aromatica*" PFBC. TR, trace (<1%); ND, not detected.

| Strains | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| $C_{12:0}$ | 2.4 | TR | ND | ND | ND |
| $C_{16:0}$ | 5.3 | 18.2 | 8.2 | 10.8 | 12.3 |
| $C_{16:0}$ 2-OH | ND | 1.8 | 3.0 | 2.0 | 3.7 |
| $C_{16:0}$ 3-OH | 6.2 | TR | TR | TR | TR |
| anteiso $C_{17:1}$ ω9c | ND | 1.1 | ND | ND | ND |
| $C_{18:0}$ | 1.5 | 2.4 | 2.3 | 2.1 | 1.4 |
| $C_{18:0}$ 3-OH | 1.8 | 1.9 | 2.0 | 3.1 | 2.5 |
| cyclo $C_{19:0}$ ω8c | 1.1 | 12.2 | 21.0 | 11.9 | 6.5 |
| *Summed features | | | | | |
| 2 | ND | 1.7 | 2.0 | 2.5 | 2.1 |
| 3 | 6.0 | 11.9 | TR | TR | TR |
| 8 | 73.4 | 44.1 | 59.9 | 65.1 | 70.2 |

*Summed features represent groups of two or more fatty acids that could not be separated using the MIDI system. Summed feature 2, $C_{14:0}$ 3-OH and/or iso $C_{16:1}$ I; summed feature 3, $C_{16:1}$ω7c and/or $C_{16:1}$ω6c; summed feature 8, $C_{18:1}$ω7c and/or $C_{18:1}$ω6c.

222

223 **Figure legend**

224 Fig. 1 Neighbor-joining tree based on 16S rRNA gene sequence of strain Ok2G$^T$ and closely related species.

225 Bootstrap percentages are represented at branch nudes. Minimum-evolution method yielded a tree of identical

226 topology.

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