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ORIGINAL PAPER

Novosphingobium aquaticum sp. nov., isolated from lake water in Suwon, Republic of Korea

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Abstract A novel Gram-stain negative, yellow coloured, strictly aerobic, rod-shaped, non-motile bacterium designated as THW-SA1^T, was isolated from lake water near Samsung apartment, Suwon, Republic of Korea. The phylogenetic analysis based on 16S rRNA gene sequences showed that strain THW-SA1^T belongs to the genus *Novosphingobium* and is closely related to *Novosphingobium taihuense* (97.8 %) and *Novosphingobium subterraneum* (97.1 %). The DNA–DNA relatedness values between strain THW-SA1^T and the most closely related type strains were found to be less than 30.0 %. The DNA G+C content was determined to be 67.5 mol%. The strain grows optimally at 25–28 °C, at pH 7.0, and in the presence of 0.5 % NaCl. The predominant isoprenoid quinone was identified as ubiquinone Q-10. The polar lipid profile comprises diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidyldimethylethanolamine, sphingoglycolipid, phosphatidylcholine, some unidentified phospholipids and some unidentified polar lipids. Fatty acids characteristic for this genus, such as $C_{16:1}$, $C_{14:0}$ 2-OH, $C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c* (summed feature 3) and $C_{18:1}$ *ω6c* and/or $C_{18:1}$ *ω7c* (summed feature 8) were also detected. On the basis of the phenotypic and genotypic analysis, the strain THW-SA1^T is considered to represent a novel species of the genus *Novosphingobium*, for which the name *Novosphingobium aquaticum* sp. nov. is proposed. The type strain is THW-SA1^T (=KCTC 42608^T=CCTCC AB 2015114^T).

Keywords *Novosphingobium aquaticum* · Ubiquinone Q-10 · Gram-stain negative · 16S rRNA

The NCBI GenBank accession number for the 16S rRNA gene sequence of strains THW-SA1^T is KP410550.

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Introduction

The genus *Sphingomonas* was first described by Yabuuchi et al. (1990), and belongs to class *Alphaproteobacteria* and family *Sphingomonadaceae*. Based

on refined phylogenetic, chemotaxonomic and physiological analyses, the genus *Sphingomonas* has been split into five genera: *Novosphingobium*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis* and *Sphingosinicella* (Takeuchi et al. 2001; Maruyama et al. 2006). The genus *Novosphingobium* was first described by Takeuchi et al. (2001) with the description of type species *Novosphingobium capsulatum.* The members of the genus are Gram-negative, aerobic, rod-shaped, motile or non-motile organisms, which form yellow to whitish brown colonies. Members of the genus contain Q-10 as the respiratory quinone and show predominance of hydroxy and unsaturated fatty acids (Takeuchi et al. 2001; Niharika et al. 2013; Glaeser et al. 2013a, b; Kämpfer et al. 2015a, b). Most species of the genus contain diphosphatidylglycerol, phosphatidylethanolamine, phosphatidymethylethanolamine, phosphatidylglycerol, sphingoglycolipid and phosphatidylcholine as polar lipids.

Members of the genus *Novosphingobium* have been isolated from a variety of habitats including soil, coastal or freshwater sediments (Balkwill et al. 1997; Sohn et al. 2004; Liu et al. 2005), surface water layers of lakes (Glaeser et al. 2009; 2013a, b), activated sludge/wastewater treatment plants (Neef et al. 1999; Fujii et al. 2003), oil-contaminated soil (Kämpfer et al. 2011), contaminated groundwater bioremediation reactors (Tiirola et al. 2002; 2005), and associated with plants (Lin et al. 2014). These bacteria play an important role in biodegradation of organic pollutants such as PAHs (Sohn et al. 2004), aromatic hydrocarbons (Yuan et al. 2009), dibenzofuran (Suzuki and Hiraishi 2007) and 2,4-dichlorophenoxyacetic (Dai et al. 2015).

At the time of writing, the genus *Novosphingobium* contains 31 species with validly published names (http://www.bacterio.net/index.html) and several new species have been recently been described, including *Novosphingobium gossypii* (Kämpfer et al. 2015), *Novosphingobium rhizosphaerae* (Kämpfer et al. 2015a, b), *Novosphingobium fluoreni* (Gao et al. 2015), *Novosphingobium marinum* (Huo et al. 2015), *Novosphingobium endophyticum* (Li et al. 2015) and *Novosphingobium tardum* (Chen et al. 2015). The aim of the present work was to determine the taxonomic position of strain THW-SA1^T.

Materials and methods

Isolation and growth conditions

Strain THW-SA1^T was isolated from lake water near Samsung apartment in Suwon, Republic of Korea. Water sample was collected in a sterile falcon tube and transferred to the laboratory. Water sample was diluted in 0.85 % (w/v) saline solution, serially diluted up to 10^{-6} and spread on nutrient agar (NA, BD, USA). The plates were incubated at 28 °C for 1 week. Single colonies were selected and transferred onto new plates for purification. Routine cultivation was performed on NA at 28 °C and the isolate was stored at −80 °C in nutrient broth (NB, BD) supplemented with 25 % glycerol. Strain THW-SA1^T has been deposited in the Korean Collection for Type Cultures (KCTC 42608^T) and China Centre for Type Culture Collection (CCTCC AB 2015114^T). For the comparative study, reference strains *Novosphingobium taihuense* KACC 18096^T and *Novosphingobium subterraneum* KCTC 2889^T were obtained the Korean Collection for Type Cultures and Korean Agricultural Culture Collection. These reference strains were cultured under the same optimum conditions as strain THW-SA1^T.

Morphological and physiological characterization

After 2 days growth on NA at 28 °C, the cells' morphology was examined. Bacterial cells were suspended in NB and were placed on carbon- and formvar-coated nickel grids for 30 s and grids were floated on one drop of 0.1 % (w/v) aqueous uranyl acetate, blotted dry and then viewed with a transmission electron microscope (Model JEM1010; JEOL) at 11,000× magnification under standard operating conditions. Gram-staining was determined using a Gram stain Kit according to the manufacturer's instructions (bioMérieux, France). Motility was determined by the hanging-drop technique (Bernardet et al. 2002). Growth at different temperatures (4, 10, 15, 18, 25, 28, 30, 35, 37 and 42 °C) was checked on NA after 7 days of incubation. Different media such as Reasoner's 2A agar (R2A; BD), tryptone soya agar (TSA, Oxoid), NA, Luria–Bertani agar (LB; Oxoid), Marine agar (MA; BD) and MacConkey agar (Oxoid) was tested for growth at 28 °C for 7 days. The salinity test was performed by using 0 to 5 % (w/v) NaCl in NB

using increments of 0.5 %. Growth of strain THW-SA1^T was also checked at different pH values, from 4.0 to 10.0 in NB using increments of 0.5 pH units. The salinity and pH tests were performed at 28 °C. The following pH buffers were used (final concentration, 100 mM): acetate buffer was used for pH 4.0–6.5 and phosphate buffer was used for pH 7.0–10.0. pH of NB was confirmed after autoclaving. Growth was estimated by monitoring the optical density at 600 nm. Anaerobic growth was tested in serum bottles containing NB supplemented with thioglycolate (0.1 %, w/v) and in which the air was substituted with nitrogen gas. Production of flexirubin-type pigments was determined by the reversible colour shift to red, purple or brown when yellow or orange colonies are covered with aqueous 20 % KOH solution (Fautz and Reichenbach 1980). Catalase activity was determined by the production of bubbles from 3 % (v/v) H_2O_2 solution mixed with freshly grown cells and oxidase activity was determined by using of 1 % (w/v) *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine reagent (Sigma, USA) according to the manufacturer's instructions. Tests for hydrolysis were performed on NA containing (w/v): casein (2 % skim milk, Oxoid, England), 1 % starch (BD), Tween 80 [0.01 % $CaCl_2{\cdot}2H_2O$ and 1 % Tween 80 (Sigma)], Tween 20 [0.01 % $CaCl_2{\cdot}2H_2O$ and 1 % Tween 20 (Sigma)], 1 % chitin (Sigma), 0.5 % L-tyrosine (Sigma), 0.1 % carboxymethyl-cellulose (CMC, Sigma), esculin (Bile esculin agar, BD) and DNA (DNase agar, Oxoid). Carbon-source assimilation and enzyme activity for novel isolate and all reference strains were conducted using API 20NE and API ZYM kits at 28 °C according to the manufacturer's instructions (bioMérieux, France). API 20NE were recorded after incubation for 48 h, under the optimal conditions for each strain while API ZYM was recorded after incubation for 10 h.

16S rRNA sequencing and phylogenetic construction

Genomic DNA was extracted and purified using a commercial Genomic DNA extraction kit (Solgent, Korea). The 16S rRNA gene was amplified using the universal bacterial primer sets including 27F/1492R (Lane 1991) and 518F/800R (Weisburg et al. 1991). The purified PCR products were sequenced by Solgent Co. Ltd (Daejeon, Korea). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and EzTaxon e-server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). Multiple alignments were performed with CLUSTAL X program (Thompson et al. 1997) and gaps were edited using the BioEdit program (Hall 1999). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura 1983). The phylogenetic trees were constructed with neighbor-joining (Saitou and Nei 1987), maximum-parsimony (Fitch 1971) and maximum-likelihood (Felsenstein 1981) methods by using the MEGA 6 program package (Tamura et al. 2013). In order to take the confidential levels for the branches (Felsenstein 1985), bootstrap analysis with 1000 replications was conducted.

G+C mol% content and DNA–DNA hybridization

For determination of the DNA G+C content, genomic DNA strain THW-SA1^T was extracted, purified according to the protocol of Moore and Dowhan (1995) and degraded enzymatically into nucleosides (nuclease P1 and alkaline phosphatase; Sigma). The nucleosides were analyzed using a reverse-phase HPLC system (Alliance 2690 system, Waters) as described previously (Mesbah et al. 1989) with reversed-phase column SunFireTM C18 (4.6 × 250 mm × 5 µm), flow rate of 1.0 ml/min, solvent mixture of 200 mM $(NH_4)H_2PO_4$/acetonitrile (97: 3, v/v) as mobile phase, and detector wavelength at 270 nm. The genomic DNA of *Escherichia coli* strain B (Sigma-Aldrich D4889) was used as a standard.

DNA–DNA hybridization was performed fluorometrically, according to the method developed by Ezaki et al. (1989) with modifications (Stabili et al. 2008), using photobiotin-labelled DNA probes and micro-dilution wells. DNA–DNA hybridization was carried out to determine levels of relatedness of the novel strain THW-SA1^T with its closest relatives *N. taihuense* KACC 18096^T and *N. subterraneum* KCTC 2889^T. The optimum renaturation temperature (45.5 °C) is calculated as [(0.51 × G+C content) + 47]—36 (Gillis et al. 1970), where 36 °C is the correction for the presence of 50 % formamide (McConaughy et al. 1969). Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage DNA–DNA relatedness values. DNA–DNA hybridization

Table 1 Physiological and biochemical characteristics of train THW-SA1^T most closely related species of the genus *Novosphingobium*

Characteristics	1	2	3
Oxidase	+	−	−
Anaerobic growth	Strictly aerobic	Aerobic	Aerobic
Hydrolysis of			
DNA	+	−	−
Starch	−	−	+
CMC	−	+	+
API 20NE			
β-Galactosidase	+	+	−
L-Arabinose	+	−	−
API ZYM			
Cystine arylamidase	−	+	−
α-Galactosidase	+	−	−
β-Galactosidase	+	+	−

Strains 1 THW-SA1^T, 2 *N. taihuense* KACC 18096^T, 3 *N. subterraneum* KCTC 2889^T. All data are from this study unless otherwise indicated. + positive, – negative

experiments were not performed with type strains of species showing 16S rRNA gene sequence similarity less than 97.0 %.

Chemotaxonomic characterization

For quinone and polar lipids, lyophilized cells of strain THW-SA1^T and *N. taihuense* KACC 18096^T were used. Respiratory quinones extracted as described previously by (Hiraishi et al. 1996; Collins and Jones 1981; Tamaoka et al. 1983) and subsequently analysed with using a reversed-phase HPLC system (Alliance 2690 system; Waters) [solvent; methanol: 2-propanol (7:5, v/v), flow rate; 1.0 ml min^{-1}]. Polar lipids of strain THW-SA1^T and reference strain *N. taihuense* KACC 18096^T were analyzed by two-dimensional TLC as described by Minnikin et al. (1984). Polar lipids extracts were spotted onto the lower left-hand corner of a thin layer plates TLC Kiesel gel 60 F_{254} plates (10 × 10 cm, Merck, USA). The plates were developed with chloroform: methanol: water (65:25:4, by vol.) in the first direction and chloroform: methanol: acetic acid: water (80:12:15:4, by vol.) in the second direction. For detection of total and specific lipids, following reagents were used 5 % molybdatophosphoric acid (total lipids, Sigma), 0.2 % ninhydrin (aminolipids, Sigma), and 2.5 % α-naphthol-sulfuric acid (glycolipids, Sigma) followed by drying at 120 °C for 5–10 min. TLC plates also sprayed with molybdenum blue reagent (Sigma) for detecting phospholipids. No heating step is needed for this reagent.

For fatty acid analysis, all strains were grown on NA at 28 °C for 48 h. Cells in exponential growth phase were used. Fatty acid were extracted, methylated and saponified by method described by Sherlock Microbial Identification system (MIDI) and analyzed by capillary GC (Hewlet Packard 6890) using the TSBA library version 6.1 (Sasser 1990).

Results and discussion

Cells of THW-SA1^T were observed to be Gram-stain negative, strictly aerobic, non-motile rods of 0.5–0.7 μm in width and 1.0–1.5 μm in length (Supplementary Fig. S1). The colonies of strain THW-SA1^T grown on NA were observed to be yellow, round, sticky with approximate diameter 2–3 mm. The comparison of biochemical and physiological characteristics between strain THW-SA1^T, *N. taihuense* KACC 18096^T and *N. subterraneum* KCTC 2889^T indicated that all strains were found to be positive for catalase; hydrolysis of Tween 80, Tween 20 and esculin; assimilation of D-glucose and D-maltose but negative for nitrate reduction, indole production, glucose acidification and arginine dihydrolase; hydrolysis of L-tyrosine, caesin, chitin, gelatin and urea; assimilation of D-mannose, D-mannitol, *N*-acetylglucosamine, gluconic acid, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid. All strains were found to be positive for the following enzyme activities alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphtol-*AS-BI*-phosphohydrolase, β-glucuronidase, α-glucosidase and β-glucosidase but negative for the following: lipase (C14), α-chymotrypsin, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The strain THW-SA1^T can be distinguished from the closest phylogenetic relatives by differences in cystine arylamidase, α-galactosidase and β-galactosidase production, hydrolysis of DNA, starch and CM; assimilation of β-galactosidase and L-arabinose. Detailed biochemical and physiological characteristics are given in Table 1. The results of the phenotypical and biochemical properties also

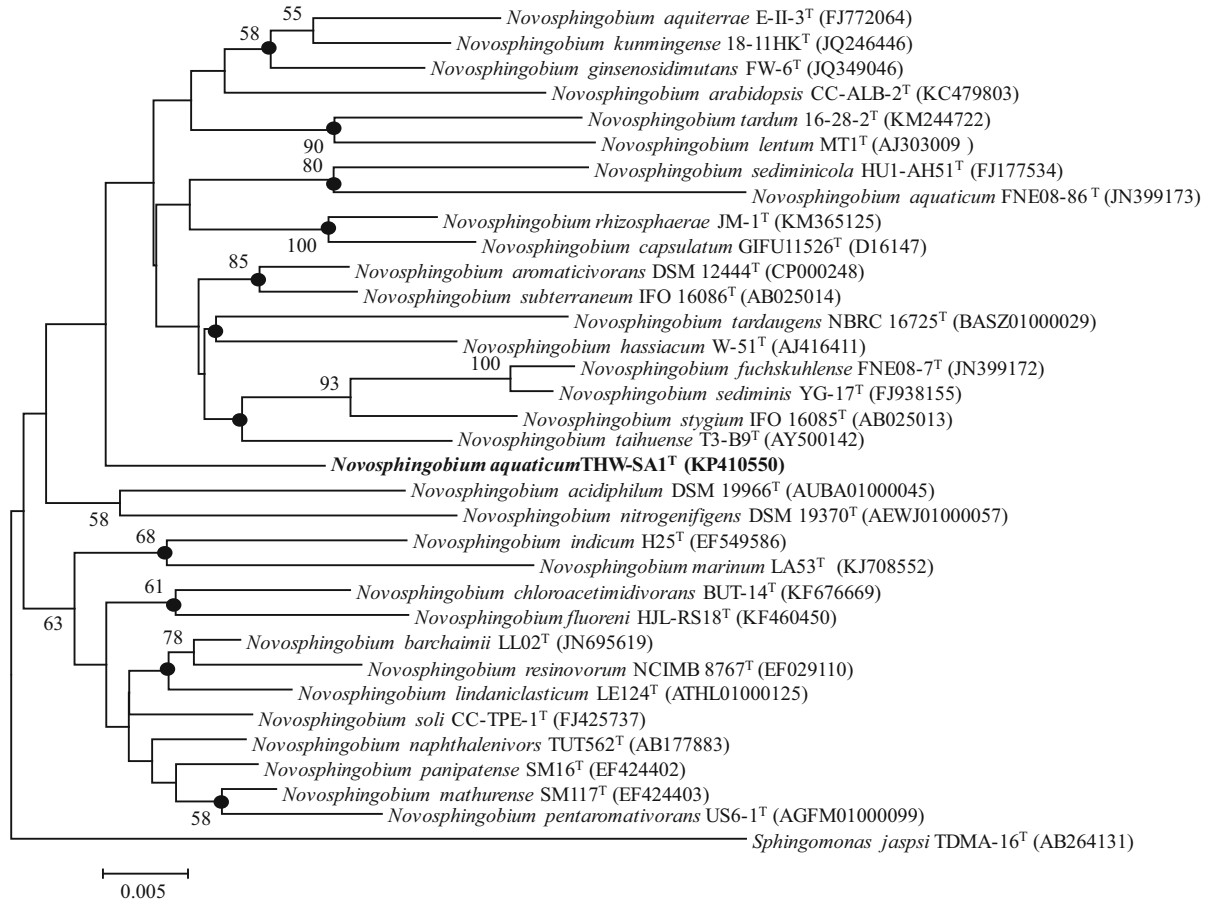

Fig. 1 The neighbor-joining tree based on 16S rRNA gene sequence analysis, showing the relationships between strain THW-SA1^T and members of the genus *Novosphingobium*. *Filled circles* indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. *Numbers* at nodes indicate bootstrap percentages (based on 1000 resampled datasets). Bootstrap values less than 50 % were not indicated. *Sphingomonas jaspsi* TDMA-16^T was used as an out group. *Scale bar* 0.005 substitutions per nucleotide position

suggested that the novel isolate represents a novel species of the *Novosphingobium*.

The 16S rRNA gene sequence analysis of strain THW-SA1^T revealed that strain THW-SA1^T belongs to the genus *Novosphingobium*. Sequence similarity calculated using the EzTaxon-e server indicated that the closest relatives of strain THW-SA1^T were *N. taihuense* KACC 18096^T (97.8 %) followed by *N. subterraneum* KCTC 2889^T (97.1 %). Neighbor-joining and maximum-likelihood phylogenetic trees based on 16S rRNA gene sequences showed that the strain THW-SA1^T is clustered within the species of the genus *Novosphingobium* (Fig. 1; Supplementary Fig. S2).

The DNA G+C content of novel isolate was identified as 67.5 mol %, which conforms to the expected range of G+C contents for the genus *Novosphingobium*. The level of DNA–DNA relatedness between strain THW-SA1^T and *N. taihuense* KACC 18096^T, *N. subterraneum* KCTC 2889^T were 28.5 ± 1 % and 23.8 ± 0.8 %, respectively. All DNA–DNA relatedness values were significantly lower than the threshold value of 70 % recommended for species delineation (Wayne et al. 1987). The results of the DNA–DNA hybridization clearly indicated that strain THW-SA1^T represents a distinct species.

Strain THW-SA1^T contains ubiquinone Q-10 as the predominant isoprenoid quinone, which is commonly detected in the members of the genus *Novosphingobium*. The polar lipids was found to

Table 2 Fatty acid profiles of strain THW-SA1^T and closely related species of the genus *Novosphingobium*

Fatty acid	1	2	3
Straight-chain			
$C_{16:0}$	14.7	18.6	16.8
$C_{18:0}$	3.8	2.9	5.8
Unsaturated			
$C_{17:1}$ $\omega6c$	Tr	Tr	2.8
$C_{17:1}$ $\omega8c$	Tr	Tr	Tr
$C_{18:3}$ $\omega6c$	Tr	Tr	1.0
$C_{18:1}$ $\omega5c$	1.5	1.0	1.0
$C_{18:1}$ $\omega7c$ 11-methyl	2.6	4.5	3.4
Hydroxyl			
$C_{14:0}$ 2-OH	12.3	14.0	11.8
$C_{15:0}$ 2-OH	Tr	Tr	1.0
Summed feature 3	24.5	19.6	14.4
Summed feature 8	35.6	34.2	36.1

Strains 1 THW-SA1^T, 2 *N. taihuense* KACC 18096^T, 3 *N. subterraneum* KCTC 2889^T. All data were obtained from the present study. Fatty acids amounting to less than 0.5 % in all strains were not listed. Summed feature 3 consisted of $C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$. Summed feature 8 consisted of $C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$

Tr trace amount (<1.0 %)

consist of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidyldimethylethanolamine (PDE), sphingoglycolipid (SGL), phosphatidylcholine (PC), some unidentified phospholipids (PL1, PL2 & PL3) and some unidentified polar lipids (L1, L2, L3 & L4). The polar lipid profile of strain THW-SA1^T and *N. taihuense* KACC 18096^T are shown in Supplementary Fig. S3. Some unidentified polarlipids (L2, L3 & L4) and an unidentified phospholipid (PL3) were detected in strain THW-SA1^T but were not present in *N. taihuense* KACC 18096^T. The major fatty acids (>10 %) of strain THW-SA1^T were found to be mainly composed of $C_{16:1}$ (14.7 %), $C_{14:0}$ 2-OH (12.3 %), $C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$ (summed feature 3; 24.5 %) and $C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$ (summed feature 8; 35.6 %). The fatty acid composition of strain THW-SA1^T was found to be very similar to that of the reference strains (Table 2).

The results of the phylogenetic analysis and the chemotaxonomic characteristics (major fatty acids, polar lipids and isoprenoid quinone) support the assignment of THW-SA1^T to the genus *Novosphingobium*. Physiological, biochemical characteristics and genomic distinctness can be used to differentiate strain THW-SA1^T from other species. Therefore the strain THW-SA1^T (=KCTC 42608^T=CCTCC AB 2015114^T) represents a novel species of the genus *Novosphingobium*, for which name *Novosphingobium aquaticum* sp. nov. is proposed.

Description of *Novosphingobium aquaticum* sp. nov

Novosphingobium aquaticum (a.qua'ti.cum. L. neut. adj. aquaticum, from water)

Cells are Gram-stain negative, strictly aerobic, rod-shaped (approximately, 0.5–0.7 μm in ×1.0–1.5 μm), non-motile, catalase and oxidase positive. On NA round, sticky and yellow pigmented colonies are produced. Growth occurs on NA, R2A, TSA and LB but not on MA and MacConkey agar. Optimum growth occurs on NA at 25-28 °C, at pH 7.0, and in the presence of 0.5 % NaCl. Hydrolysis of DNA, Tween 80, Tween 20 and esculin is positive but casein, chitin, L-tyrosine, starch and CMC is negative. Flexirubin-type pigments are absent.

According to API 20NE test, positive for aesculin hydrolysis, β-galactosidase and assimilation of L-arabinose, D-glucose and D-maltose; negative for nitrate reduction, indole production, glucose acidification, arginine dihydrolase, gelatinase, urease and assimilation of D-mannose, D-mannitol, *N*-acetylglucosamine, gluconic acid, capric acid, adipic acid, malate, trisodium citrate and phenylacetic acid. In API ZYM tests, positive results are obtained for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphtol-*AS-BI*-phosphohydrolase, β-glucuronidase, α-glucosidase, β-glucosidase, α-galactosidase and β-galactosidase; negative results are obtained for lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Ubiquinone Q-10 is the predominant isoprenoid quinone. The polar lipid profile contains of DPG, PG, PE, PDE, SGL, PC, PL1-3 and L1-4. The fatty acid profile consists of major amounts of $C_{16:1}$, $C_{14:0}$ 2-OH, $C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$ (summed feature 3) and $C_{18:1}$ $\omega6c$ and/or $C_{18:1}$ $\omega7c$ (summed feature 8). The DNA G+C content of the type strain is 67.5 mol%.

The type strain is THW-SA1^T (=KCTC 42608^T=CCTCC AB 2015114^T), which was isolated from lake water near Samsung apartment in Suwon, Republic of Korea.

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