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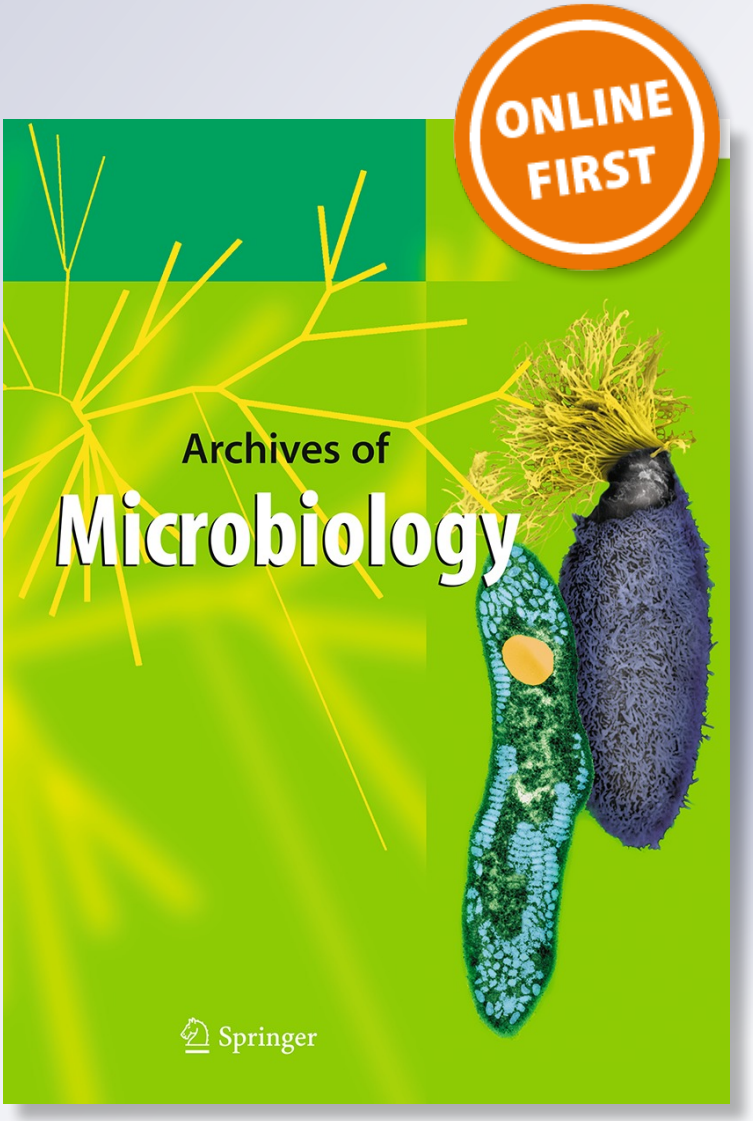

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ORIGINAL PAPER

Acinetobacter plantarum sp. nov. isolated from wheat seedlings plant

Juan Du[1] · Hina Singh[1] · Hongshan Yu[2] · Feng-Xie Jin[2] · Tae-Hoo Yi[1]

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Abstract Strain THG–SQM11^T, a Gram-negative, aerobic, non-motile, coccus-shaped bacterium, was isolated from wheat seedlings plant in P. R. China. Strain THG–SQM11^T was closely related to members of the genus *Acinetobacter* and showed the highest 16S rRNA sequence similarities with *Acinetobacter junii* (97.9 %) and *Acinetobacter kookii* (96.1 %). DNA–DNA hybridization showed 41.3 ± 2.4 % DNA reassociation with *A. junii* KCTC 12416^T. Chemotaxonomic data revealed that strain THG–SQM11^T possesses ubiquinone-9 as the predominant respiratory quinone, $C_{18:1}\omega9c$, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), and $C_{16:0}$ as the major fatty acids. The major polar lipids were found to be diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine. The DNA G+C content was 41.7 mol %. These data, together with phenotypic characterization, suggest that the isolate represents a novel species, for which the name *Acinetobacter plantarum* sp. nov. is proposed, with THG–SQM11^T as the type strain (=CCTCC AB 2015123^T =KCTC 42611^T).

Keywords *Acinetobacter plantarum* · 16S rRNA · Ubiquinone-9

Introduction

The genus *Acinetobacter* belongs to the family *Moraxellaceae* in the class *Gammaproteobacteria*. Members of the genus *Acinetobacter* are characterized as Gram-negative, oxidase-negative, catalase-positive, and non-motile coccobacilli. The organisms can grow over a wide temperature range on usual culture media and have the ability to survive under a wide range of environmental conditions for extended periods of time (Juni 1972; Choi et al. 2013; Feng et al. 2014). Nine genomic species of the genus *Acinetobacter* with provisional designations were initially delineated solely on the basis of DNA–DNA hybridization. Recently, several of these have been characterized more extensively and are now validly published species of the genus (Dijkshoorn et al. 2007; Nemec et al. 2010; Li et al. 2014b). At the time of writing, the genus *Acinetobacter* contains more than 40 validly published species. These *Acinetobacter* species are distributed ubiquitously in nature, including in clinical specimens, activated sludge, soil, compost, plant, raw milk, raw wastewater, lake water, and river water (Nishimura et al. 1988; Carr et al. 2003; Nemec et al. 2003; Vaneechoutte et al. 2008; Anandham et al. 2010; Vaz-Moreira et al. 2011; Li et al. 2014a; Nemec et al. 2015). In this study, a new bacterium, designated strain THG–SQM11^T, was characterized by a polyphasic taxonomic approach.

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The NCBI GenBank accession numbers for the 16S rRNA gene sequence and *rpoB* gene sequence of strain THG–SQM11^T are KM598254 and KR856237, respectively.

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Materials and methods

Isolation of bacterial strain

Sample was collected from wheat seedlings on August 16, 2014 in Shangqiu (34°22′40″N 115°39′43″E), Henan province, P. R. China. Sample was suspended in sterile saline solution 0.85 % (w/v), serially tenfold-diluted, and spread on nutrient agar (NA, Oxoid). The plates were incubated at 28 °C for 1 week. Single colonies were purified by transferring to new NA. Strain THG–SQM11^T was cultured routinely on NA at 28 °C and maintained with 25 % (v/v) glycerol at −70 °C. Strains *Acinetobacter junii* KCTC 12416^T (*A. junii* KCTC 12416$^{T)}$ and *Acinetobacter kookii* KCTC 32033^T (*A. kookii* KCTC 32033^T), obtained from Korean Collection for Type Cultures (KCTC) were used as reference strains and cultured as same conditions of strain THG–SQM11^T.

Morphological and physiological characterization

Gram reaction was determined by using bioMérieux (France) Gram stain kit. After culturing for 24 h at 28 °C on NA, cells from single colony were suspended, placed on carbon- and formvar-coated nickel grids for 30 s, and stained with 0.1 % (w/v) aqueous uranyl acetate then observed by transmission electron microscope (Model JEM1010; JEOL) at ×11,000 magnification under standard operating conditions. Motility was tested in sulfide-indole motility medium (SIM; Difco). Anaerobic growth was performed in NB supplemented with thioglycollate [0.1 % (w/v)], for the air was substituted with nitrogen gas. Growth at different temperatures (4, 10, 15, 18, 25, 28, 30, 35, 42 and 50 °C) was tested in NB and estimated by monitoring the optical density at 600 nm after 5 days of incubation at 28 °C. Production of oxidase and catalase activity were tested using cells grown on NA for 24 h, with 1 % (w/v) *N*, *N*, *N*′, *N*′-tetramethyl-1,4-phenylenediamine reagent and 3 % (v/v) H_2O_2, respectively. Tests for hydrolysis were performed on NA containing: casein [2.0 % (w/v) skim milk, Oxoid]; starch [1.0 % (w/v), Difco], CM-cellulose (CMC) [1.0 % (w/v), Sigma]; L-tyrosine [0.5 % (w/v), Sigma]; chitin [1.0 % (w/v), Sigma]; Tween 20 [1.0 % (w/v), Sigma]; Tween 80 [1.0 % (w/v), Sigma]; and DNA (DNase agar, Oxoid). Growth on tryptone soya agar (TSA, Oxoid), Luria–Bertani agar (LA, Oxoid), Reasoner's 2A agar (R2A agar; Difco), Marine agar (MA, Oxoid), and MacConkey Agar (Oxoid) was also tested. In the above description, except specially indicated, all tests were evaluated after 7 days of incubation at 28 °C. In addition, API 20NE, API 32GN, and API ZYM tests (bioMérieux) were carried out to evaluate basic chemical test, carbon-source utilization, and enzyme activities on the bases of the instructions of the manufacturer.

Molecular characterization and phylogenetic construction

Genomic DNA of strain THG–SQM11^T was prepared as described previously (Moore and Dowhan 1995). The 16S rRNA gene was amplified from the chromosomal DNA with the universal bacterial primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Weisburg et al. 1991), and the purified PCR products were sequenced by Solgent Co. Ltd (Daejeon, Korea). Sequences of related taxa were obtained from the EzTaxon-e server (http://www.ezbiocloud.net/eztaxon; Kim et al. 2012) and GenBank database. Multiple alignments were performed via program CLUSTAL_X (Thompson et al. 1997). Gaps were edited in the BioEdit program (Hall 1999). Phylogenetic tree was constructed with neighbor-joining method (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981), and maximum-parsimony (Fitch 1971) methods by using the MEGA 6 program package (Kumar et al. 2008; Tamura et al. 2013). The Kimura two-parameter model (Kimura 1983) was used to calculate the evolutionary distances. The bootstrap values were calculated based on 1,000 replications (Felsenstein 1985).

Comparative sequence analysis of *rpoB* gene was performed for strains THG–SQM11^T and currently described species of the genus *Acinetobacter*. Two variable regions of the *rpoB* gene: Zone 1, encompassing nucleotide positions 2916–3267, and Zone 2, encompassing nucleotide positions 3263–3773 were amplified and analyzed as described by La Scola et al. (2006) and Nemec et al. (2009). Primers Ac696F (5′-TAYCGYAAAGAYTTGAAAGAAG-3′) and Ac1093R (5′-CMACACCYTTGTTMCCRTGA-3′) were used to amplified Zone 1; primers Ac1055F (5′-GTGATAARATGGCBGGTCGT-3′) and Ac1598R (5′-CGBGCRTGCATYTTGTCRT-3′) were used to amplified Zone 2. Purified PCR products were sequenced by SolGent Co. Ltd, using the aforementioned primers.

The DNA G+C content was analyzed using reverse-phase HPLC system (Alliance 2690 system, Waters) as described by Mesbah et al. (1989). The genomic DNA of *Escherichia coli* strain B (Sigma-Aldrich D4889) was used as a standard. DNA–DNA hybridization was performed between strains THG–SQM11^T and *A. junii* KCTC 12416^T, using fluorometrically with photobiotin-labelled probes in microplate wells (Ezaki et al. 1989). 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.

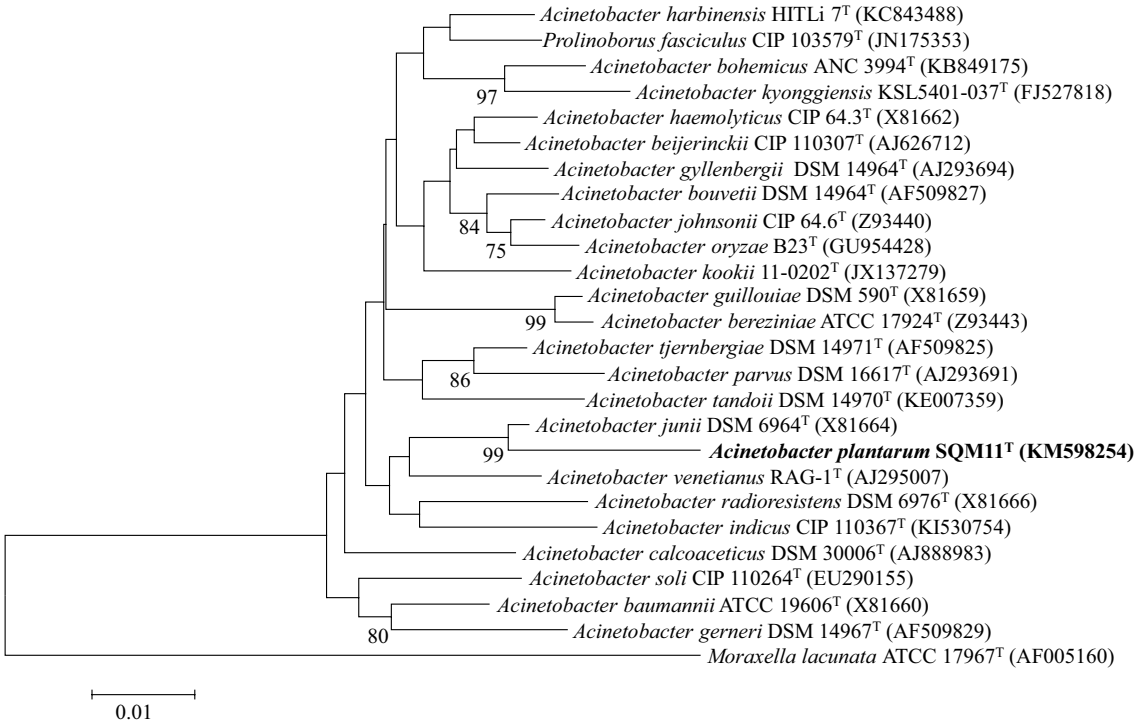

Fig. 1 Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationships of strain THG–SQM11^T with related *Acinetobacter* species. Bootstrap values (expressed as percentage of 1000 replications) over 70 % are shown at branch points. *Moraxella lacunata* ATCC 17967^T (AF005160) was used as outgroup. *Bar*, 0.01 substitutions per nucleotide position

Chemotaxonomic characterization

For the cellular fatty acid analysis, cells of strain THG–SQM11^T, *A. junii* KCTC 12416^T and *A. kookii* KCTC 32033^T, were cultured on NA for 2 days at 28 °C (the type strains exhibited similar growth rates), and then biomass from the third quadrant of each plate was collected and used for the preparation (http://www.microbialid.com/PDF/TechNote_101.pdf). The cellular fatty acid profiles were prepared according to the protocol of Sherlock Microbial Identification System (MIDI) and identified with GC (Hewlett Packard 6890) using Sherlock Aerobic Bacterial Database (TSBA60) (Sasser 1990). For the extraction of polar lipids and respiratory quinones of strain THG–SQM11^T and related species, cells were cultured in NB for 2 days and freeze-dried after harvesting. The polar lipids of strain THG–SQM11^T and *A. junii* KCTC 12416^T were extracted (Minnikin et al. 1977, 1984) and detected using two-dimensional thin-layer chromatography (Tindall 1990). For the presence of total and specific lipids, following reagents were used: 5 % molybdophosphoric acid (total lipids, Sigma); 0.2 % ninhydrin (aminolipids, Sigma); molybdenum blue (phospholipids, Sigma); 2.5 % α-naphthol-sulfuric acid (glycolipids, Sigma). Respiratory quinones were extracted and analyzed by RP-HPLC Waters 2690 Alliance system as previously described (Collins and Jones 1981; Hiraishi et al. 1996).

Results and discussion

The almost complete 16S rRNA gene sequence of strain THG–SQM11^T (1452 bp) was analyzed. Sequence similarity indicated that the closest phylogenetic neighbors of strain THG–SQM11^T were *A. junii* KCTC 12416^T (97.9 %) and *A. kookii* KCTC 32033^T (96.1 %). Strain THG–SQM11^T shown lower than 96.0 % similarities to other members of the genus *Acinetobacter*. The phylogenetic trees also indicated that strain THG–SQM11^T was clearly affiliated with the genus *Acinetobacter* and formed a phyletic line distinct from the clades of related species (Fig. 1 and Supplementary Fig. S1).

The partial *rpoB* gene sequence (861 bp) of strain THG–SQM11^T showed the highest similarity with *A. junii* (98.6–97.8 %) and lower than 87.0 % similarities to other members of the genus *Acinetobacter*. Neighbor-joining phylogenetic tree based on the *rpoB* gene (Supplementary Fig. S2) showed that strain THG–SQM11^T clustered only with *A. junii* (EU477110). The DNA G+C content of strain THG–SQM11^T was 41.7 mol %. Strain THG–SQM11^T showed 41.3 ± 2.4 % DNA–DNA

relatedness with *A. junii* KCTC 12416^T. These low DNA relatedness values suggested that THG–SQM11^T as a novel species of genus *Acinetobacter* (Stackebrandt and Goebel 1994).

Strain THG–SQM11^T was Gram negative, non-motile, aerobic, oxidase negative, and catalase-positive. The isolate grew well on Reasoner's 2A agar (R2A agar), tryptone soya agar (TSA), Luria–Bertani agar (LA), Marine agar (MA), and MacConkey agar, and grew over a wide temperature range (10–42 °C) on nutrient agar (NA). These characters demonstrated that strain THG–SQM11^T is in line with the genus *Acinetobacter*. Strain THG–SQM11^T was coccus-shaped (Supplementary Fig. S3), while species of the genus *Acinetobacter* were characterized by coccobacillary rods (Choi et al. 2013). Additional physiological and biochemical characteristics of strain THG–SQM11^T are summarized in the species description and a comparison of strain THG–SQM11^T and related type strains is given in Table 1.

The major cellular fatty acids (>10.0 %) of strain THG–SQM11^T were $C_{18:1}\omega9c$ (34.1 %), summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 22.4 %), and $C_{16:0}$ (19.0 %). The fatty acid composition of strain THG–SQM11^T was similar to *A. junii* KCTC 12416^T, showed only small quantitative differences, but showed some more differences to *A. kookii* KCTC 32033^T (Table 2). Strain THG–SQM11^T contained Q–9 as the predominant respiratory quinone. The polar lipid profiles of strain THG–SQM11^T and *A. junii* KCTC 12416^T are shown in Supplementary Fig. S4. Diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylcholine (PC), two unidentified aminolipids (AL1–2), and three unidentified lipids (L1–3) were detected on THG–SQM11^T. The polar lipid profiles of strain THG–SQM11^T was distinguishable from that of *A. junii* KCTC 12416^T by the absence of one unidentified aminolipids (AL3) and contained different unidentified lipids (L3 in THG–SQM11^T and L0 in *A. junii* KCTC 12416^T.

The characteristics of the novel isolate are consistent with the description of the genus *Acinetobacter* with regard to morphological, biochemical, and chemotaxonomic properties. On the basis of phylogenetic distances between strain THG–SQM11^T and recognized *Acinetobacter* species indicated by 16S rRNA gene and *rpoB* gene sequence, the values of DNA–DNA hybridization between strain THG–SQM11^T and the closely related species, the combination of unique phenotypic characteristics (Table 1), respiratory quinone, the fatty acid profile and polar lipid profile, strain THG–SQM11^T represents a novel species of the genus *Acinetobacter*, for which the name *Acinetobacter plantarum* sp. nov. is proposed.

Table 1 Physiological characteristics of strain THG–SQM11^T and related type strains of species of genus *Acinetobacter*

Characteristic	1	2	3
Assimilation of			
D-Glucose	−	+	−
D-Melibiose	−	+	−
D-Sorbitol	+	−	−
D-Ribose	−	+	−
D-Maltose	+	−	−
D-Sucrose	+	−	−
L-Fucose	+	−	−
L-Histidine	+	+	−
L-Rhamnose	−	+	−
L-Alanine	+	−	+
L-Serine	W	+	+
Lactose	−	+	+
Citric acid	+	+	−
Malic acid	+	+	−
Salicin	−	+	−
Inositol	−	+	−
Propionate	−	+	+
Phenylacetic acid	−	−	+
N-acetyl-glucosamine	−	+	−
3-hydroxy-butyrate	+	−	−
Enzyme activity			
Lipase (C14)	+	−	−

Strains: 1. THG–SQM11^T ; 2. *A. junii* KCTC 12416^T; 3. *A. kookii* KCTC 32033^T. All data was carried out in this study with same culture condition. All strains are positive for alkaline phosphatase, esterase lipase (C8), esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase and hydrolysis of Tween 20, Tween 80, L-tyrosine; negative for hydrolysis of casein, starch, DNA, CMC and chitin, nitrate reduction, glucose acidification, indole production, arginine dihydrolase, protease, urease, trypsin, β-galactosidase, α-fucosidase, α-glucosidase, β-glucosidase, α-galactosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-chymotrypsin and β-glucuronidase. All strains are positive for assimilation of: capric acid, sodium acetate, glycogen, valerate, and L-proline; negative for assimilation of: D-glucose, L-arabinose, D-mannose, D-Mannitol, gluconate, adipic acid, sodium malonate, *N*-acetyl-glucosamine, 2-ketogluconate, 5-ketogluconate, 4-hydroxy-benzoate, 3-hydroxy-benzoate, suberate and itaconate

+ Positive, – negative, *w* weakly positive

Description of *Acinetobacter plantarum* sp. nov

Acinetobacter plantarum (plan.ta'rum. L. n. planta a sprout; L. gen. pl. n. *plantarum* of plants).

Cells are Gram negative, aerobic, non-motile, oxidase negative, catalase positive, and coccus shaped (approximately 0.80 μm in diameter; Supplementary Fig. S3).

Table 2 Cellular fatty acid profiles of strain THG–SQM11^T and phylogenetically related species of the genus *Acinetobacter*

Fatty acid	1	2	3
$C_{10:0}$	2.1	1.9	1.2
$C_{12:0}$	0.1	ND	13.7
$C_{14:0}$	0.6	0.7	0.7
$C_{16:0}$	19.0	22.0	16.1
$C_{17:0}$	1.2	1.1	0.5
$C_{18:0}$	2.1	2.4	3.4
$C_{16:1}$ ω9c	0.7	0.6	ND
$C_{17:1}$ ω8c	2.2	1.6	1.2
$C_{18:1}$ ω7c	1.0	0.8	4.0
$C_{18:1}$ ω9c	34.1	31.2	20.2
$C_{12:0}$ 2OH	4.6	4.6	0.2
$C_{12:0}$ 3OH	6.4	5.2	6.3
Anteiso–$C_{11:0}$	0.5	0.3	0.6
Anteiso–$C_{12:0}$	0.4	0.7	0.7
Anteiso–$C_{15:0}$	0.2	ND	0.8
Summed feature 3*	22.4	24.2	28.5

Summed feature 3* consisted of $C_{16:1}$ ω7c/$C_{16:1}$ ω6c

Strains: 1. THG–SQM11^T ; 2. *A. junii* KCTC 12416^T; 3. *A. kookii* KCTC 32033^T. All the data were taken from this work. Fatty acids of <0.5 % in all strains were not listed

ND not detected

Colonies on NA are circular, smooth, pale white, and convex 0.7–1.0 mm in diameter after 2 days of incubation at 28 °C. Growth occurs at 10–42 °C (optimum 25–35 °C). Cells grow well on NA, TSA, MA, LA, R2A agar, and MacConkey agar. Tween 20, Tween 80, and L-tyrosine are hydrolyzed but casein, starch, DNA, CMC, or chitin are not. Positive for following enzyme activities: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. The following substrates can be utilized as sole carbon source: malic acid, citric acid, capric acid, D-sorbitol, valerate, L-fucose, L-histidine, 3-hydroxy-butyrate, L-proline, D-maltose, D-sucrose, L-alanine, L-serine, sodium acetate, and glycogen. The major fatty acids are $C_{18:1}\omega 9c$, summed feature 3 ($C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$), and $C_{16:0}$. The predominant respiratory quinone is Q–9. The G + C content of genomic DNA is 41.7 mol %. The composition of polar lipids is DPG, PE, PG, PC, AL1–2, and L1–3.

The type strain, THG–SQM11^T (=CCTCC AB 2015123^T = KCTC 42611^T), was isolated from wheat seedlings in Shangqiu, P. R. China.

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