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NOTE

Alicyclobacillus tengchongensis sp. nov., a Thermo-Acidophilic Bacterium Isolated from Hot Spring Soil[§]

Min Goo Kim[1,2], Jae-Chan Lee[3], Dong-Jin Park[2], Wen-Jun Li[4,5], and Chang-Jin Kim[1,2*]

[1]*Department of Biomolecular Science, University of Science and Technology, Daejeon 305-350, Republic of Korea*
[2]*Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea*
[3]*Institute of Microbial Ecology and Resources, Mokwon University, Daejeon 302-729, Republic of Korea*
[4]*The Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, Yunnan, P. R. China*
[5]*Key Laboratory of Biogeography and Bioresource in Arid Land, Chinese Academy of Science, Xinjiang Institute of Ecology and Geography, Chinese Acedemy of Sciences, Ürümqi 830011, Xinjiang, P. R. China*

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A thermo-acidophilic bacterium, designated strain ACK006^T, was isolated from the soil of a hot spring at Tengchong in China. Cells were Gram-staining-positive, motile, catalase-positive and oxidase-negative, spore-forming rods. The isolate grew aerobically at 30–50°C (optimum at 45°C), pH 2.0–6.0 (optimum pH 3.2) and 0–5.0% (w/v) NaCl (optimum 1% NaCl). Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain ACK006^T belongs to the genus *Alicyclobacillus* with the sequence similarity of 92.3, 92.4, 92.5, and 92.8% to *Alicyclobacillus cycloheptanicus* SCH^T, *Alicyclobacillus ferrooxydans* TC-34^T, *Alicyclobacillus contaminans* 3-A191^T and *Alicyclobacillus disulfidooxidans* SD-11^T, respectively. Similarity to other species of the genus *Alicyclobacillus* was 90.3–92.8% and similarity to species of the genus *Tumebacillus* was 85.9–87.8%. The genomic DNA G+C content was 53.7 mol%. The predominant menaquinone was MK-7. Major fatty acids were ω-cycloheptane $C_{18:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:0}$. The cell-wall peptidoglycan was the A1γ type; containing *meso*-diaminopimelic acid as the diagnostic diamino acid. On the basis of polyphasic analysis from this study, strain ACK006^T represents a novel species of the genus *Alicyclobacillus* for which the name *Alicyclobacillus tengchongensis* sp. nov. is proposed. The type strain is ACK006^T (=KCTC 33022^T =DSM 25924^T).

Keywords**:** *Alicyclobacillus tengchongensis* sp. nov., acidophilic bacterium, taxonomy

The genus *Alicyclobacillus* was first proposed to separate three *Bacillus* species that contained ω-alicyclic fatty acids and were phylogenetically distant from other members of *Bacillus* (Wisotzkey *et al*., 1992). Subsequently, the genus was emended by Goto *et al*. (2003) and Karavaiko *et al*. (2005) to include species that did not contain ω-alicyclic fatty acids and to extend the temperature growth range and DNA G+C content range. At the time of writing, the genus *Alicyclobacillus* contains 21 species with validly published names and two subspecies (Euzéby, 2013). Members of the genus *Alicyclobacillus* are Gram-positive, thermo-acidophilic, heterotrophic organisms that have often been found in extreme habitats such as hot springs [*Alicyclobacillus vulcanalis* (Simbahan *et al*., 2004) and *Alicyclobacillus ferrooxydans* (Jiang *et al*., 2008)], geothermal soil [*Alicyclobacillus pohliae* (Imperio *et al*., 2008)] and acidic environments [*Alicyclobacillus acidocaldarius* (Wisotzkey *et al*., 1992), *Alicyclobacillus acidiphilus* (Matsubara *et al*., 2002), and *Alicyclobacillus acidoterrestris* (Deinhard *et al*., 1987)].

During the course of a study to screen thermo-acidophilic bacteria, strain ACK006^T was isolated from the soil of a hot spring located in Tengchong County (24° 38′–25° 52′ N 98° 05′–98° 46′ E) which is the main volcanogeothermal region in Yunnan Province, China. It was isolated using the dilution plating technique on a solid WAYE (washed agarose/yeast extract) medium (Johnson, 1995) followed by aerobic incubation at 45°C for two days. The strain was stored at -80°C with 20% (v/v) glycerol. In order to characterize strain ACK006^T phenotypically, the isolate was routinely grown aerobically on *Bacillus caldarius* medium (BAM, Deinhard *et al*., 1987) for three days at 45°C and pH 3.2, except where indicated otherwise.

The morphology of the isolate was observed by Gram staining and transmission electron microscopy using cells from exponentially growing cultures. Motility was observed by light microscopy (Nikon Eclipse 80i) and the hanging drop method. The presence of spores was determined by a specific spore-staining test, using malachite green (Shaeffer and Fulton spore stain kit; Sigma). Spore formation was enhanced by growing the bacteria on BAM agar at 45°C for more than seven days. The flagellum type was determined

*For correspondence. E-mail: changjin@kribb.re.kr; Tel.: +82-42-860-4332; Fax: +82-42-860-4625
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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain ACK006^T is KF772795.

by transmission electron microscopy using cells from the exponential growth phase. Cells were mounted on Formvar-coated copper grids and negatively stained with 1% potassium phosphotungstate (pH 7.0). Gram staining was performed by the Hucker method (Murray *et al*., 1994). Catalase, oxidase and nitrate reduction, hydrolysis of esculin, gelatin, starch and urea and production of indole were tested as recommended by Smibert and Krieg (1994). Acid formation from carbon compounds was determined using the method described by Deinhard *et al*. (1987) with the API 50 CH kit (bioMérieux, France). When acidification was ambiguous, strains were cultivated in BAM basal salts medium with 0.2% carbon compounds added and the pH indicator omitted. After cultivation, a decrease in the pH values of cultured broths was measured with a pH meter. Carbon compounds that gave pH values lower than that of the control (i.e., culture without carbon compounds) by 0.4 or more were scored as positive. Enzyme activities were tested using API ZYM kit system according to the instructions of the manufacturer (bioMérieux). To determine the optimal temperature and pH for growth, the strain were incubated in BAM broth at different temperatures (4, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60°C) and pH range of 0.5–7.0 (in increments of 0.5 pH units, adjusted by the addition of 1 M H_2SO_4). NaCl tolerance was tested in BAM broth at 0–10% (w/v) NaCl (at 1% intervals). Growth was monitored by turbidity at OD_{600} using a spectroscopic method (model UV-1650PC; Shimadzu). Susceptibility to antibiotics was tested on BAM plates using antibiotic discs containing the following: amikacin, 30 μg; amoxicillin, 10 μg; ampicillin, 20 μg; bacitracin, 10 U; carbenicillin, 100 μg; cefotaxime, 30 μg; cefoxitin, 30 μg; cephalexin, 30 μg; chloramphenicol, 10 μg; ciprofloxacin, 5 μg; colistin sulphate, 10 μg; doxycycline, 30 μg; erythromycin, 15 μg; gentamicin, 10 μg; kanamycin, 30 μg; lincomycin, 15 μg; methicillin, 5 μg; nalidixic acid, 30 μg; neomycin, 30 μg; nitrofurantoin, 300 μg; norfloxacin, 10 μg; novobiocin, 30 μg; nystatin, 100 μg; oxacillin, 1 μg; penicillin, 10 U; piperacillin, 75 μg; polymixin B, 100 U; rifampicin, 30 μg; streptomycin, 10 μg; teicoplanin, 30 μg; tetracycline, 30 μg; tobramycin, 10 μg; and vancomycin, 30 μg.

Genomic DNA from strain ACK006^{T} was prepared using a commercial genomic DNA-extraction kit (Solgent, Korea). The 16S rRNA gene was amplified by PCR with the forward primer Eubac 27F and the reverse primer 1492R (DeLong, 1992). Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3730XL; Applied Biosystems). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR, USA). The 16S rRNA gene sequences of related taxa were obtained from the GenBank and EzTaxon servers and the identification of phylogenetic neighbors and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://www.eztaxon-e.ezcloud.net/) (Kim *et al*., 2012). The 16S rRNA gene sequence was aligned with the published sequences of closely related bacteria using CLUSTAL W 2.1 software (Larkin *et al*., 2007). Gaps at the 5′ and 3′ ends of the alignment were omitted from further analyses. Phylogenetic trees were constructed using three different methods: the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1972) algorithms within the MEGA5 program (Tamura *et al*., 2011).

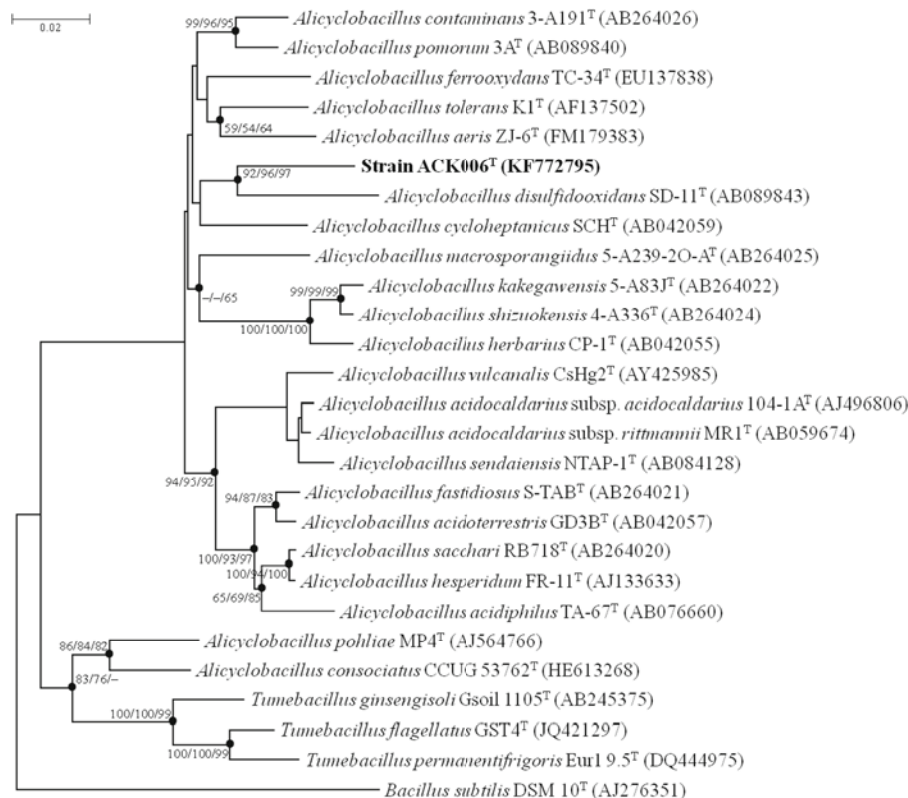

Fig. 1. Rooted neighbor-joining tree based on 16S rRNA gene sequences of strain ACK006^{T} and all related type species of the genera *Alicyclobacillus* and *Tumebacillus*. Filled circles at nodes indicate generic branches that were also recovered using maximum-likelihood and maximum-parsimony algorithms. Bootstrap values, expressed as a percentage of 1,000 replications, are given at branching points as calculated by neighbor-joining/maximum-likelihood/maximum-parsimony probabilities; when >50%. *Bacillus subtilis* DSM 10^{T} was used as an outgroup. Bar, 0.02 substitutions per nucleotide position.

Evolutionary distance matrices for the neighbor-joining method were calculated using the algorithm of the Kimura 2-parameter model (Kimura, 1980). To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1,000 replications) was performed (Felsenstein, 1985). The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from GenBank and their strain designations and accession numbers are shown in Fig. 1.

Table 1. Differential characteristics of strain ACK006^T and related type strains of the genus *Alicyclobacillus*

Strains: 1, Strain ACK006^T; 2, *Alicyclobacillus disulfidooxidans* DSM 12064^T; 3, *Alicyclobacillus cycloheptanicus* KCTC 3458^T; 4, *Alicyclobacillus contaminans* DSM 17975^T; 5, *Alicyclobacillus ferrooxydans* DSM 22381^T. All strains are negative for nitrate reduction and for acid production from erythritol, D-adonitol, *N*-acetylglucosamine, inulin, D-melezitose, D-raffinose, xylitol, gentiobiose, L-fucose and D-arabitol. All strains are positive for the acid production from esculin. Symbols: +, positive; –, negative; ND, not determined; w, weakly positive.

Characteristics	1	2	3	4	5
Origin	Hot spring soil	Wastewater sludge	Car service station soil	Orange juice	Solfataric soil
Cell size (μm)	0.5–0.7 × 2.0–3.5	0.6–1.0 × 1.0–6.0	0.4–0.6 × 2.5–4.5	0.8–0.9 × 4.0–5.0	0.4–0.6 × 2.5–4.5
Growth factor	Yeast extract	Yeast extract	Methionine, vitamin B12, pantothenate, isoleucine	Not required	Yeast extract
G+C content (mol%)	53.7	53.0	55.0	60.3	48.6
Growth temp. (°C) range (opt.)	30–50 (45)	4–40 (35)	40–53 (48)	35–60 (50–55)	17–40 (28)
Growth pH range (opt.)	2.0–6.0 (3.2)	0.5–6.0 (1.5–2.5)	3.0–5.5 (3.5–4.5)	3.0–6.0 (4.0–4.5)	2.0–6.0 (3.0)
Growth at 5% NaCl	+	ND	+	–	–
Motility	+	–	–	+	–
Catalase	+	–	+	–	+
Oxidase	–	–	+	–	+
Hydrolysis of:					
Gelatin	+	–	–	+	–
Starch	+	+	–	–	+
Acid production from:[a]					
Glycerol	+	+	–	+	–
D-Arabinose	–	+	+	–	–
L-Arabinose	–	+	+	+	+
D-Ribose	–	–	+	+	+
L-Xylose	–	+	+	–	–
D-Xylose	+	+	+	+	–
Methyl-β-D-xylopyranoside	–	–	–	–	+
D-Galactose	–	–	–	+	–
D-Glucose	+	–	+	+	+
D-Fructose	–	–	+	+	+
D-Mannose	w	–	+	+	+
L-Sorbose	–	+	+	+	–
L-Rhamnose	+	–	+	+	–
Inositol	–	–	+	–	–
D-Mannitol	–	+	+	+	–
D-Sorbitol	–	–	+	–	+
Methyl-α D-mannopyranoside	–	–	–	–	+
Methyl-α D-glucopyranoside	+	–	–	–	+
Amygdalin	–	+	+	–	–
Arbutin	w	–	–	+	–
L-Salicin	w	–	–	+	–
D-Cellobiose	+	+	–	+	–
D-Maltose	+	+	–	+	–
D-Lactose	–	–	–	+	–
D-Melibiose	+	+	–	–	–
D-Sucrose	+	+	–	+	–
D-Trehalose	+	–	–	+	+
Glycogen	+	–	–	–	–
D-Turanose	+	–	–	–	+
D-Lyxose	–	–	+	–	+
D-Tagatose	–	+	+	+	+
D-Arabitol	–	–	+	–	–
Potassium 5-keto-gluconate	–	+	+	–	+

Data are from this study (for strain ACK006^T) or from Goto *et al.* (2007) and Jiang *et al.* (2008)

[a]Data from this study

For fatty acid analyses, different cultivation conditions (culture media and temperature) had to be used: strain ACK006^T, *A. cycloheptanicus* KCTC 3458^T and *A. contaminans* DSM 17975^T [BAM medium, 45°C], *A. disulfidooxidans* DSM 12064^T [9K medium (Silverman and Lundgren, 1959), 35°C] and *A. ferrooxydans* DSM 22381^T [Norris solid medium (Norris *et al*., 1996), 28°C]. This was because there was no single growth condition that allowed growth of all of the strains under comparison. The biomass of each strain was harvested after three days of growth. Cellular fatty acids were extracted and analyzed by GC (Agilent Technologies 6890N) according to the standard protocol of the Sherlock Microbial Identification System (version 6.1; MIDI database TSBA6). For the analysis of quinones, cells were harvested in the late-exponential phase and freeze-dried. Isoprenoid quinones were extracted and analyzed by HPLC (Shimadzu SPD-10AV), as described by Collins and Jones (1981). Isolation of DNA (Saito and Miura, 1963) and determination of the DNA G+C contents were performed by HPLC (Shimadzu SPD-10AV), as described by Mesbah *et al*. (1989). Analysis of the cell-wall peptidoglycan was carried out by the Identification Service of the DSMZ, Braunschweig, Germany. Reference strains included *Alicyclobacillus disulfidooxidans* DSM 12064^T, *Alicyclobacillus contaminans* DSM 17975^T, *Alicyclobacillus ferrooxydans* DSM 22381^T, and *Alicyclobacillus cycloheptanicus* KCTC 3458^T, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) and the Korean Collection for Type Culture (KCTC; Daejeon, Korea).

Strain ACK006^T was Gram-positive, motile with one polar flagellum, rods and colonies were circular and ivory-brown colored when grown for three days at 45°C on BAM (Supplementary data Fig. S1). Endospores are positioned terminally with swollen sporangia (Supplementary data Fig. S2). It was able to grow at 30–50°C, at pH 2.0–6.0 and at 0–5.0% (w/v) NaCl. Optimal growth was observed at 45°C; at pH 3.2. Cells were catalase-positive and oxidase-negative. Other physiological and biochemical properties and enzymatic activities of strain ACK006^T were compared to the type strains of the other species in the genus *Alicyclobacillus*. Strain ACK006^T showed a range of typical phenotypic properties of members of the genus *Alicyclobacillus* (Wisotzkey *et al*., 1992). Differentiating characteristics of strain ACK006^T, in comparison with closely related type strains, are shown in Table 1; and other physiological and biochemical properties are in the species description.

The almost-complete 16S rRNA gene sequence (1,460 bp) of strain ACK006^T was obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. Phylogenetic analysis based on the neighbor-joining algorithm revealed that strain ACK006^T and *Alicyclobacillus disulfidooxidans* SD-11^T formed a distinct and stable phyletic line in the genus *Alicyclobacillus* with high bootstrap value (>90%) (Fig. 1). The 16S rRNA sequence similarity with the other *Alicyclobacillus* species was in the range of 90.3 to 92.8%. Maximum-likelihood and maximum-parsimony methods resulted in the same tree topologies showing >95% bootstrap value with the closest type strains in the genus *Alicyclobacillus*, and only neighbor-joining results are shown. As a result of the low 16S rRNA gene-sequence similarities (<93%) to all other species of the genus with validly published names, DNA–DNA hybridizations were not performed. Major fatty acids in strain ACK006^T were ω-cycloheptane $C_{18:0}$ (52.4%), iso-$C_{17:0}$ (15.5%), and anteiso-$C_{17:0}$ (14.2%). These are common, characteristic features of members of the genus *Alicyclobacillus.* The presence of iso-$C_{17:0}$ and anteiso-$C_{17:0}$ with relatively moderate percentage distinguished strain ACK006^T from the type strains of the other species of the genus *Alicyclobacillus* (Table 2). The predominant isoprenoid quinone of strain ACK006^T was menaquinone-7 (MK-7). The diagnostic cell-wall diamino acid was *meso*-diaminopimelic acid and the peptidoglycan type was A1γ *meso*-Dpm-direct (A31 according to www.peptidoglycan-types.info). It contained the amino acids *meso*-diaminopimelic (*meso*-Dpm) acid, alanine and glutamic acid. The G+C content of the DNA of strain ACK006^T was 53.7 mol%.

Based on the above phylogenetic, genomic and phenotypic analyses, it is clear that strain ACK006^T belongs to the genus *Alicyclobacillus.* The characteristics that differentiate strain ACK006^T from the type strains of the other species of the genus *Alicyclobacillus* are summarized in Table 1. The most distinctive features were that strain ACK006^T is motile, hydrolyzes gelatin and contains ω-alicyclic fatty acids. Other differences include the acid production from glycogen but not from D-tagatose. The major cellular fatty acids are also significantly different from those of other *Alicyclobacillus* species.

Therefore, on the basis of this polyphasic taxonomic evidence, we propose that this strain represents a novel species of the genus *Alicyclobacillus*, for which the name *Alicyclobacillus tengchongensis* sp. nov. is proposed; with the type strain ACK006^T.

Table 2. Cellular fatty acid composition (%) of strain ACK006^T and related type strains of the genus *Alicyclobacillus*

Strains: 1, Strain ACK006^T; 2, *Alicyclobacillus disulfidooxidans* DSM 12064^T; 3, *Alicyclobacillus cycloheptanicus* KCTC 3458^T; 4, *Alicyclobacillus contaminans* DSM 17975^T; 5, *Alicyclobacillus ferrooxydans* DSM 22381^T. All data are from this study. –, Not detected

Fatty acids	1	2	3	4	5
$C_{14:0}$	–	–	1.0	–	–
$C_{15:0}$	–	–	0.6	–	–
$C_{16:0}$	1.0	0.9	2.2	1.3	–
$C_{17:0}$	0.6	–	–	0.9	–
$C_{18:0}$	–	–	1.1	0.9	–
iso-$C_{15:0}$	6.7	0.7	0.7	2.5	9.2
iso-$C_{16:0}$	7.0	11.5	1.3	16.9	25.3
iso-$C_{17:0}$	15.5	0.8	2.9	29.8	–
iso-$C_{18:0}$	0.2	–	–	4.7	–
anteiso-$C_{15:0}$	1.9	2.1	–	0.5	39.5
anteiso-$C_{17:0}$	14.2	–	1.2	44.3	27.7
ω-Cyclohexane $C_{17:0}$	–	43.2	–	–	–
ω-Cyclohexane $C_{19:0}$	–	7.5	–	–	–
ω-Cycloheptane $C_{18:0}$	52.4	–	86.4	–	–
ω-Cycloheptane $C_{18:0}$ 2-OH	0.4	–	2.6	–	–

Description of *Alicyclobacillus tengchongensis* sp. nov.

Alicyclobacillus tengchongensis (teng.chong.en′sis. N.L. masc. adj. *tengchongensis* of Tengchong, China, the source of the soil sample from which the type strain was isolated).

Cells are Gram-staining-positive, motile, rods, 0.5–0.7 μm × 2.0–3.5 μm in size. Endospores are terminally positioned with swollen sporangia. Colonies are circular, ivory-brown colored on BAM agar after three days incubation at 45°C. Growth occurs at 30–50°C (optimally at 45°C), pH 2.0–6.0 (optimally at pH 3.2) and 0–5.0% (w/v) NaCl (optimally at 1.0% NaCl). Catalase-positive and oxidase-negative. Esculin, gelatin and starch are hydrolyzed, but casein and urea are not. Nitrate is not reduced to nitrite. Indole is not produced. Acid is produced from arbutin, esculin, D-glucose, glycerol, salicin, D-cellobiose, D-maltose, D-melibiose, D-sucrose, D-trehalose, glycogen, D-galactose, D-turanose, D-xylose, and methyl-α-D-glucoside, but not from adonitol, dulicitol, erythritol, D-fructose, inositol, D-mannitol, D-mannose, D-sorbitol, methyl-α-D-mannopyranoside, methyl-β-D-xylopyranoside, *N*-acetylglucosamine, amygdalin, D-lactose, inulin, D-melezitose, D-raffinose, L-sorbose, starch, xylitol, gentiobiose, D-lyxose, D-tagatose, L-rhamnose, D- or L-arabinose, D- or L-fucose, D- or L-arabitol, D-ribose, L-xylose, 2-keto-gluconate, and 5-keto-gluconate. Enzyme activity is observed for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, crystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosamininase, but not for lipase (C14), β-galactosidase, β-glucuronidase, α-fucosidase and α-mannosidase (API ZYM). Susceptible to amikacin, ampicillin, lincomycin, nalidixic acid, neomycin, vancomycin, colistin sulfate, doxycycline, methicillin, nitrofurantoin, norfloxacin and novobiocin, but resistant to kanamycin, gentamicin, streptomycin, penicillin, erythromycin, amoxicillin, rifampicin, nystatin, bacitracin, tetracycline, chloramphenicol, carbenicillin, cefotaxime, cefoxitin, cephalexin, ciprofloxacin, oxacillin, piperacillin, polymixin B, and tobramycin. The major fatty acids are ω-cycloheptane $C_{18:0}$, iso-$C_{17:0}$ and anteiso-$C_{17:0}$. The predominant menaquinone is MK-7. The diagnostic cell-wall diamino acid is *meso*-diaminopimelic acid and the peptidoglycan type is A1γ ; containing the amino acids *meso*-diaminopimelic acid, alanine and glutamic acid. The DNA G+C content of the type strain is 53.7 mol%.

The type strain, ACK006^T (=KCTC 33022^T =DSM 25924^T), was isolated from soil of a hot spring in Tengchong, China.

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