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ORIGINAL PAPER

Microbacterium horti sp. nov., a bacterium isolated from *Cucurbita maxima* cultivating soil

Shahina Akter[1] · Jae Hee Park[2] · Chang Shik Yin[3]

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Abstract A novel bacterial strain THG-SL1^T was isolated from a soil sample of *Cucurbita maxima* garden and was characterized by using a polyphasic approach. Cells were Gram-reaction-positive, non-motile and rod-shaped. The strain was aerobic, catalase positive and weakly positive for oxidase. Phylogenetic analysis based on 16S rRNA gene sequence analysis but it shared highest similarity with *Microbacterium ginsengisoli* KCTC 19189^T (96.6 %), indicating that strain THG-SL1^T belongs to the genus *Microbacterium*. The DNA G + C content of the isolate was 68.9 mol %. The major fatty acids were anteiso-C15: 0 (39.7 %), anteiso-C17: 0 (24.4 %) and iso-C16: 0 (18.5 %). The major polar lipids of strain THG-SL1^T were phosphatidylglycerol (PG) and an unidentified glycolipid (GL). The predominant respiratory isoprenoid quinones were menaquinone-11 and menaquinone-12. The diamino acid in the cell-wall peptidoglycan was ornithine. Based on the results of polyphasic characterization, strain THG-SL1^T represented a novel species within the genus *Microbacterium*, for which the name *Microbacterium horti* sp. nov. is proposed. The type strain is THG-SL1^T (=KACC 18286^T=CCTCC AB 2015117^T).

Keywords *Microbacterium horti* · Gram-reaction-positive · Polyphasic taxonomy · 16S rRNA

Introduction

The genus *Microbacterium*, belonging to the family *Microbacteriaceae*, phylum *Actinobacteria*, was first described by Orla-Jensen (1919) and emended by Collins et al. (1983) and by Takeuchi and Hatano (1998). Members of the genus *Microbacterium* are short rods, which are generally aerobic and catalase positive (Collins and Bradbury 1992). The major polar lipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and unidentified glycolipids (GL), and the G + C content of the DNA ranges from 61 to 75 % (Alves et al. 2014, Takeuchi and Hatano 1998). Members of the genus *Microbacterium* play different role in the environment such as β-glucosidase-producing (Park et al. 2008, Kim et al. 2010), D-aminoacylase-producing (Liu et al. 2005), plant-growth-promoting (Madhaiyan et al. 2010), polysaccharide-producing (Matsuyama et al. 1999, Yokota et al. 1993), xylanolytic (Park et al. 2006), crude-oil-degrading (Schippers et al. 2005) and can be isolated from various habitats, including air (Zlamala et al. 2002), clinical samples, deep-sea sediments (Shivaji et al. 2007),

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✉ Chang Shik Yin
acuyin@khu.ac.kr

[1] Department of Oriental Medicinal Material and Processing College of Life Science, Kyung Hee University, 1 Seocheon, Kihung Yongin, Kyunggi 446-701, Republic of Korea

[2] Graduate School of Biotechnology, Kyung Hee University, 1 Seocheon, Kihung Yongin, Kyunggi 446-701, Republic of Korea

[3] Department of Acupuncture Meridian Science Research Center, College of Korean Medicine, Kyung Hee University, # 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea

soil (Anand et al. 2012, Kook et al. 2014, Mondani et al. 2013), sea water (Kim et al. 2008), marine environments, milk products and plant, insect and human specimens. At the time of writing, the genus *Microbacterium* comprises 90 species with validly published names (http://www.bacterio.net/microbacterium.html).

Methods and materials

Bacterial isolation

A soil sample was collected from *Cucurbita maxima* cultivating garden, Yongin-Si, Republic of Korea (37° 24′ 62″ N 127° 07′ 84″ E). About one gram of soil was homogenized in 10 ml of 0.85 % sterile saline, serially diluted, and 100 µl of aliquot was spread on nutrient agar (NA, Oxoid) as described by Liu et al. (2006). The plates were incubated at 28 °C for 2–3 days, and one light yellow-colored strain, designated as THG-SL1^T, was isolated and purified by transferring onto new plates. Strain THG-SL1^T was preserved in glycerol solution (25 %, w/v) at −80 °C and routinely cultured at 28 °C on NA medium. Strain THG-SL1^T was deposited in the China Centre for Type Culture Collection (CCTCC AB 2015117^T) and Korean Agricultural Culture Collection (KACC 18286^T). For the comparative study, the reference type strains *Microbacterium ginsengisoli* KCTC 19189^T and *Microbacterium pumilum* JCM 14902^T (Table 1) were obtained from Korean Collection for Type Cultures (KCTC) and Japan Collection of Microorganisms (JCM) culture collection centers.

Cell growth, physiology, morphology, biochemical characteristics

Cell morphology was observed at 11,000× magnification with a transmission electron microscope (JEM1010; JEOL) after negative staining with 0.5 % (w/v) uranyl acetate using cells grown for 2 days at 28 °C on NA plate. Motility was checked on sulfide-indole-motility medium (SIM, Difco). The Gram-staining test was performed by using a Gram stain kit (bioMérieux) according to the manufacturer's instructions. Growth on R2A agar (R2A, BD), NA, tryptone soy agar (TSA, Difco), Luria–Bertani agar (LA, Difco), marine agar (MA, BD) and MacConkey agar (BD) was also assessed. Growth at various temperatures (4, 10, 18, 25, 28, 30, 35, 37, 40 and 45 °C) and at various pH, ranging from pH 4.0 to 10.0 (in intervals of 0.5 pH units), was determined. For the pH tests, two different 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. The following pH buffers were used to adjust pH values: $C_2H_3NaO_2/C_2H_4O_2$ (pH 4.0–6.5), Na_2HPO_4/NaH_2PO_4 (pH 6.5–8.0) and Na_2HPO_4/NaOH (pH 8.0–10.0). Salt tolerance was determined in nutrient broth supplemented with 0.0–5.0 % (w/v) NaCl (at intervals of 0.5 % NaCl). Growth was estimated for both pH and salt tolerance by monitoring the optical density at 600 nm. After cells were grown for 2 days at 28 °C on NA, catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined by using 1.0 % (w/v) *N, N, N′, N′*-tetramethyl-1, 4-phenylenediamine reagent. Reduction of nitrate was tested in nitrate broth containing 0.2 % KNO_3 (Skerman 1967). Indole production was analyzed using Kovács's reagent in 1.0 % tryptone broth (Skerman 1967). Anaerobic growth was tested in serum bottles containing nutrient broth supplemented with thioglycolate (0.1 %), in which the air was substituted with nitrogen gas. Tests for hydrolysis were performed on NA containing: gelatin [12.0 % gelatin, (Sigma)]; esculin [0.1 % (w/v) esculin and 0.05 % (w/v) ferric citric acid, (Sigma)]; casein [2.0 % (w/v) skim milk, Oxoid]; starch [1.0 % (w/v), Difco], DNA (DNase agar, Oxoid); 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]; and Tween 80 [1.0 % (w/v), Sigma]. In the above description, all tests were evaluated after 7 days of incubation at 28 °C, except otherwise indicated. In order to study carbon source utilization and enzyme activity, API 20NE, API 32 GN and API ZYM kits were used according to the manufacturer's instructions (bioMérieux).

16S rRNA gene sequence and phylogenetic analysis

Extraction of genomic DNA was performed by a commercial genomic DNA extraction kit (Solgent, Republic of Korea). Amplification of the 16S rRNA gene was carried out with the universal primers 27F and 1492R (Lane, 1991), and the purified PCR products were sequenced by Solgent Co. Ltd (Daejeon, Republic of Korea). The 16S rRNA gene sequence of strain THG-SL1^T was compiled with SeqMan software (DNASTAR) and edited in the BioEdit program (Hall 1999). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012). In the subsequent phylogenetic analysis, CLUSTAL X software (Thompson et al. 1997) was used to align the 16S rRNA gene sequence of the novel strain with those of closely related members of the genus *Microbacterium*. Genetic distances were calculated using Kimura's two-parameter model (Kimura 1983). Phylogenetic trees were constructed with the neighbor-joining (Saitou and Nei 1987), maximum-likelihood (Takahashi and Nei 2000) and maximum-parsimony (Fitch 1971) methods by using the

Table 1 Biochemical and physiological characteristics of strain THG-SL1^T and its phylogenetic neighbors of genus *Microbacterium*

Characteristics	1	2	3
Habitat	Garden soil	Ginseng field soil	Soil
Colony color	Light yellow	Yellow	Pale yellow.
Aerobic/facultative anaerobic	Aerobic	Strictly aerobic	Aerobic
Oxidase	w	+	+
Urease	–	–	w
Growth temperature (°C)	18–37	18–30	17–32
Hydrolysis of			
Casein	w	–	w
Starch	+	–	–
L-tyrosine	–	–	+
Chitin	w	–	+
CMC	+	w	+
Tween 20	w	+	+
Tween 80	w	–	–
Enzyme activities			
Alkaline phosphatase	+	w	+
Lipase (C14)	w	–	w
Cystine arylamidase	+	+	w
Trypsin	+	+	w
α-chymotrypsin	+	–	+
Naphthol-AS-BI-phosphohydrolase	+	–	+
α-galactosidase	+	–	+
β-galactosidase	+	+	+
β-glucuronidase	w	–	+
V-acetyl-β-glucosaminidase	+	+	–
α-mannosidase	+	–	–
Assimilation of			
Arabinose	w	–	+
Mannose	w	+	+
Mannitol	+	+	–
Potassium gluconate	–	+	–
Malic acid	w	+	–
Trisodium citrate	–	–	+
Phenylacetic acid	–	–	+
Rhamnose	–	+	–
Inositol	–	+	–
Acetate	–	+	+
Alanine	–	+	–
Glycogen	+	–	–
Serine	–	+	–
Salicin	w	–	–
Melibiose	–	–	+
Propionate	–	w	+
Valerate	–	+	+
3-Hdroxybutyric acid	–	w	+
4-Hydroxybenzoic acid	–	–	+

Table 1 continued

Characteristics	1	2	3
Proline	–	w	+
Major fatty acids	Anteiso-C15: 0, anteiso-C17: 0, iso-C16: 0	Anteiso-C17: 0, Anteiso-C15: 0, iso-C16: 0	Anteiso-C15: 0, iso-C16: 0, Anteiso-C17: 0

Strains: 1, THG-SL1^T ; 2, *Microbacterium ginsengisoli* KCTC 19189^T; 3, *Microbacterium pumilum* JCM 14902^T

All data were obtained from this work. All strains were positive for: catalase; hydrolysis of esculin and DNAse; enzyme activities of esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, α-glucosidase, β-galactosidase and β-glucosidase; fermentation and assimilation of glucose; assimilations of maltose and sucrose. All strains were negative for: hydrolysis of gelatin; enzyme activity of α-fucosidase; reduction of nitrates and indole production; assimilation of arginine dihydrolase, *N*-acetyl-glucosamine, capric acid, adipic acid, ribose, itaconate, suberate, malonate, lactate, ketogluconate, 3-hydroxybenzoic acid, fucose, sorbitol or histidine. +, positive; w, weakly positive; -, negative

MEGA5 program (Tamura et al. 2011). In order to determine the confidence levels for the branches (Felsenstein 1985), bootstrap analysis with 1,000 replications was also conducted.

G + C content

For the measurement of DNA G + C content, genomic DNA was extracted and purified as described by 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 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.

Chemotaxonomic characteristics

Respiratory quinone and polar lipids

The freeze-dried cells for the analysis of polar lipids and isoprenoid quinones were produced from cultures grown for 4 days in nutrient broth at 28 °C. Respiratory quinone of isolate THG-SL1^T and reference strain *M. ginsengisoli* KCTC 19189^T was extracted using 300 mg lyophilized cells with chloroform: methanol (2: 1, v/v), separated by using hexane and eluted with hexane: diethyl ether (98: 2, v/v); then, eluent was evaporated by rotatory evaporator and dissolved in acetone, according to Collins (1985). Menaquinone purification was determined using a reverse-phase HPLC system (Alliance 2690 system, Waters) [wavelength: 270 nm, solvent MeOH: isopropanol (7: 5, v/v), flow rate: 1.0 ml/min] as previously described Hiraishi et al. (1996).

Polar lipids of strain THG-SL1^T and *M. ginsengisoli* KCTC 19189^T were examined by two-dimensional TLC (Merck Kiesel gel 60 F254 plates, 10,610 cm) and identified as described by Minnikin et al. (1977). The mixture of chloroform/methanol/water (65:25:4, by vol.) was used as developed solvent for the first dimension and the mixture of chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) as developed solvent for the second dimension. For detection of total and specific lipids, the plates developed in the solvent system were sprayed with 5.0 % molybdophosphoric acid for total lipids, 0.2 % ninhydrin for aminolipids, molybdenum blue reagent (Sigma) for phospholipids and 2.5 % α-naphthol-sulfuric acid for glycolipids, followed by drying at 120 °C.

Peptidoglycan analysis

Peptidoglycan analysis of strain THG-SL1^T and reference strain *M. ginsengisoli* KCTC 19189^T was analyzed as mentioned by Schleifer and Kandler (1972). In 50 mM phosphate buffer, 200 mg of freeze-dried cells was dissolved. After 3 times sonication, cell fractions were removed by centrifugation and supernatant was collected in new tube. Again, after centrifugation step, supernatant was discarded and pellets collected. The pellets were dissolved in 4 % SDS solution and kept in 100 °C ovens until it became colorless. The pellets were collected by centrifugation, washed three times by distilled water and again centrifuged. Then peptidoglycan samples were hydrolyzed by 6 N HCl at 100 °C. The hydrolyzed peptidoglycans were centrifuged, and then, supernatants were filtered. HCl was evaporated, and dried fragments were finally dissolved in water. The hydrolyzed peptidoglycans were determined by spotting the sample on the cellulose TLC plate [TLC cellulose Merck KGaA (20 × 20 cm)].

Cellular fatty acid analysis

For the analysis of fatty acids, cells of strain THG-SL1^T and reference strains, *M. ginsengisoli* KCTC 19189^T, *Microbacterium pumilum* JCM 14902^T, were cultured on

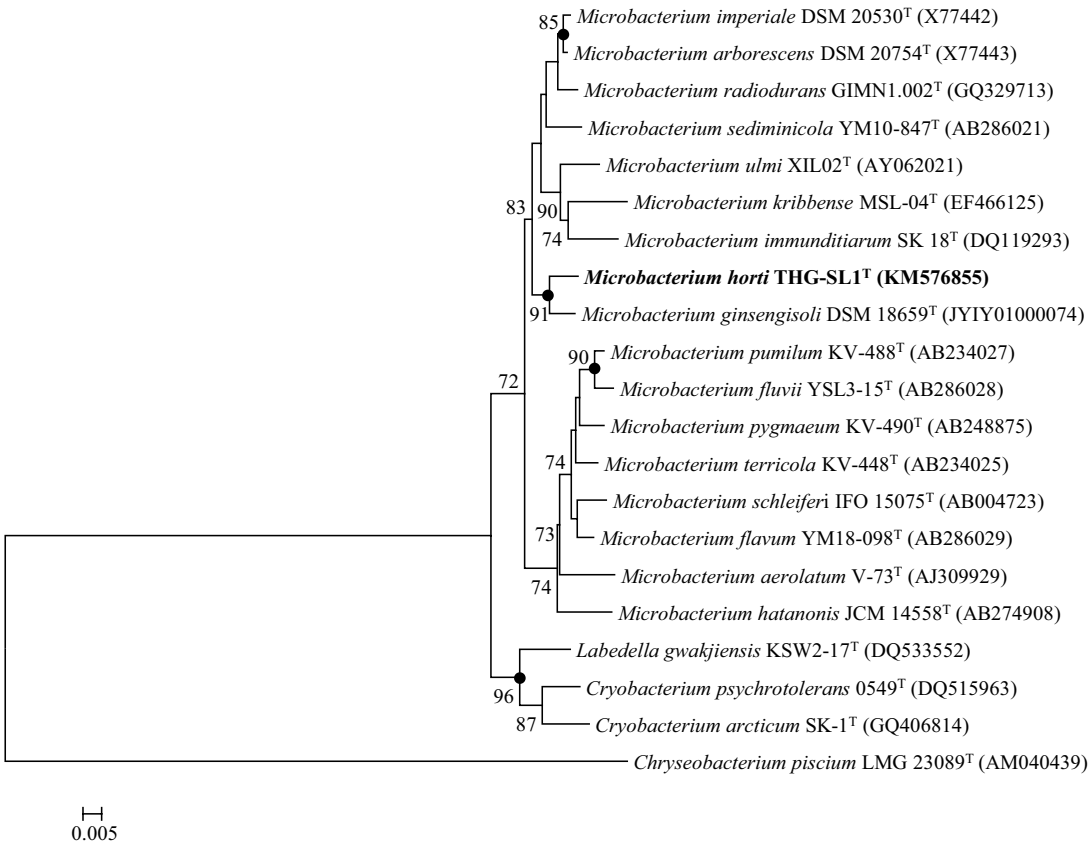

Fig. 1 Neighbor-joining (NJ) tree based on 16S rRNA gene sequence analysis showing phylogenetic relationships of strain THG-SL1^T and members of the genus *Microbacterium*. *Filled circles* indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. Bootstrap values more than 70 % based on 1000 replications are shown at branching points. *Scale bar*, 0.005 substitutions per nucleotide position

NA plates at 28 °C for 2 days, respectively; 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 acids were analyzed by gas chromatography (model 6890; Hewlett Packard) using the Microbial Identification software package with the Sherlock system MIDI 6.1 and the Sherlock Aerobic Bacterial Database (TSBA 6.1) (Sasser 1990).

Results and discussion

Cell growth, morphology, physiology and biochemical tests

Strain THG-SL1^T was aerobic, Gram-reaction-positive, catalase positive, weakly positive for oxidase, non-motile and rod-shaped. Colonies on NA were circular after incubation at 28 °C for 2 days. The isolate grew on R2A agar, NA, TSA, LB agar and MA, but did not on MacConkey agar. Strain THG-SL1^T grew at temperature 18–37 °C (optimum 28 °C); at pH 6.0–8.5 (optimum, pH 7.5) and 0–1.5 % NaCl (w/v) (optimum 0.5 % NaCl), respectively. The isolate hydrolyzed starch, CMC and DNase but did not hydrolyze L-tyrosine. Other biochemical and physiological characteristics of strain THG-SL1^T and references strain are shown in Table 1. The negative properties of API ZYM, API 20NE and API 32 GN tests of strain THG-SL1^T are listed in Supplement Table 1.

16S rRNA gene sequence and phylogenetic analysis

The nearly complete sequence (1436 bp) of the 16S rRNA gene was obtained. The comparison of the 16S rRNA gene sequence of strain THG-SL1^T (NCBI GenBank accession number KM576855) with other *Microbacterium* strains indicated that strain THG-SL1^T is a novel strain of the genus *Microbacterium*, sharing distant sequence similarity with *M. ginsengisoli* KCTC 19189^T (96.6 %) and *M. pumilum* JCM 14902^T (96.0 %). Strain THG-SL1^T formed an

Table 2 Cellular fatty acid profiles of strain THG-SL1^T and the reference strains of genus *Microbacterium*

Fatty acid	1	2	3
Iso-C14: 0	1.4	1.1	2.5
Iso-C15: 0	5.2	3.4	3.9
Anteiso-C15: 0	39.7	30.5	44.8
C16: 0	1.9	tr	3.9
Iso-C16: 0	18.5	19.9	24.7
Iso-C17: 0	2.7	1.3	4.5
Anteiso-C17: 0	24.4	40.1	11.1
C18: 0	1.2	tr	3.5
Iso-C18: 0	1.3	1.5	tr

Strains: 1, THG-SL1^T ; 2, *Microbacterium ginsengisoli* KCTC 19189^T; 3, *Microbacterium pumilum* JCM 14902^T

All data were obtained from this work. Fatty acids of less than 0.5 % in all strains are not listed. tr: traces (<1.0 %)

individual lineage with *M. ginsengisoli* KCTC 19189^T in the neighbor-joining tree (Fig. 1), maximum-likelihood tree (Supplementary Fig. S2) and the maximum-parsimony tree (data not shown).

DNA G + C content mol % analysis

The DNA G + C content mol % of strain THG-SL1^T was 68.9 %, which is within the range for members of the genus *Microbacterium* (Park et al. 2008). As low sequence similarities (<97.0 %) were found between strain THG-SL1^T and all species of the genus *Microbacterium* with validly published names, DNA–DNA hybridization studies of strain THG-SL1^T with its close relatives were not performed, following the recommendation of Stackebrandt and Goebel (1994).

Fatty acid, polar lipid, quinone and peptidoglycan analysis

The major fatty acids (>10 %) were found anteiso-C15: 0 (39.7 %), anteiso-C17: 0 (24.4 %) and iso-C16: 0 (18.5 %) in strain THG-SL1^T. The amount of fatty acid iso-C16: 0 was less than its closest type strains (Table 2). The isolated strain THG-SL1^T showed a similar major fatty acid composition to its two relatives, but there were significant quantitative differences when cultivated under the same conditions (Table 2). Thus, the results indicate that strain THG-SL1^T is a new species in the genus *Microbacterium*. The cellular fatty acid profile of strain THG-SL1^T and the reference strains is shown in Table 2. The major polar lipids of strain THG-SL1^T were phosphatidylglycerol (PG) and an unidentified glycolipid (GL). The isolate also contained two unidentified lipids (L1-2) (Supplementary Fig. S2), which have been reported in the several members of the genus *Microbacterium*. The major isoprenoid respiratory quinone of the strain THG-SL1^T was MK-11 and MK-12 that were similar to other members of genus *Microbacterium*. The peptidoglycan of strain THG-SL1^T and reference strain *M. ginsengisoli* KCTC 19189^T contained ornithine in the cell-wall peptidoglycan (Supplementary Fig. S4), thus appearing to belong to peptidoglycan-type B2, like the majority of *Microbacterium* type strains (Schleifer and Kandler 1972).

The physiological, biochemical and morphological characteristics of strain THG-SL1^T are given in the species description and Table 1. Phenotypic examination revealed many traits in common between the novel strain and the other species of the genus *Microbacterium*. However, strain THG-SL1^T could be differentiated clearly from its closest reference type strains by the ability to hydrolyze starch and by showing positive activity of α-mannosidase. The fatty acid profiles (Table 2) and 16S rRNA gene sequence analysis suggested the placement of strain THG-SL1^T as novel strain in the genus *Microbacterium*. This phylogenetic inference is supported by the unique combination of chemotaxonomic and biochemical characteristics of the novel strain. Hence, strain THG-SL1^T constitutes a member of the genus *Microbacterium* and the name of the strain THG-SL1^T (=KACC 18286^T =CCTCC AB 2015117^T) *Microbacterium horti* sp. nov. is proposed.

Description of *Microbacterium horti* sp. nov

Microbacterium horti (hor'ti. L. masc. n. hortus garden, horti from the garden)

Cells are Gram-reaction-positive; catalase and oxidase positive and weakly positive, respectively, non-motile, aerobic and rod-shaped. Colonies are circular, light yellow-colored on NA. Cells grow on NA, R2A, TSA, LA and MA but not on MacConkey agar. Growth occurs at 18–37 °C (optimum 28 °C), at pH 6.0–8.5 (optimum pH 7.5) and up to 0.0–1.5 % NaCl (w/v) (optimum 0.5 % NaCl). Gelatin or L-tyrosine is not hydrolyzed. Starch, esculin, CMC and DNase are hydrolyzed. Casein, chitin, Tween 20 and Tween 80 are weakly hydrolyzed. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase and α-mannosidase activities are present, weakly positive for lipase (C14) and β-glucuronidase activities and positive for fermentation and assimilation of glucose. Maltose, mannitol, sucrose and glycogen are assimilated. Arabinose, mannose, malic acid and salicin are weakly assimilated.

The diamino acid in the cell-wall peptidoglycan is ornithine. The major predominant isoprenoid quinone and polar lipid of the strain THG-SL1^T are MK-11 and MK-12 and PG and GL, respectively. The major fatty acid is anteiso-C15: 0, anteiso-C17: 0 and iso-C16: 0. The DNA G + C content mol % of the type strain is 68.9 %.

The type strain is THG-SL1^T (=KACC 18286^T =CCTCC AB 2015117^T), and it was isolated from *Cucurbita maxima* cultivating soil, Republic of Korea.

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