Deinococcus hibisci sp. nov., isolated from rhizosphere of Hibiscus syriacus L. (mugunghwa flower)

Gabriela Moya,¹ Zheng-Fei Yan,¹ Dong-Hun Chu,¹ KyungHwa Won,¹ Jung-Eun Yang,¹ Qi-Jun Wang,² Moo-Chang Kook³ and Tae-Hoo Yi¹,*

Abstract

A Gram-stain-positive, pink-pigmented, coccus-shaped, strictly aerobic, non-motile bacterium, strain THG-AG1.5T, was isolated from rhizosphere of Hibiscus syriacus L. (Mugunghwa flower) located in Kyung Hee University, Yongin, Gyeonggi, Republic of Korea. The isolated strain grew optimally at 25–30°C, at pH 6.0–7.5 and in the presence of additional 0–1.5% (w/v) NaCl. Strain THG-AG1.5T exhibited tolerance to UV radiation (>1500 J m⁻²) and to gamma radiation (>12 kGy). Based on 16S rRNA gene sequence comparisons, strain THG-AG1.5T was closely related to Deinococcus daejeonensis MJ27T (98.03%), Deinococcus radiotolerans C1T (97.61%) and Deinococcus grandis DSM 3963T (97.32%). The genomic DNA G+C content of strain THG-AG1.5T was 74.8 mol%. The DNA–DNA hybridization values between strain THG-AG1.5T and its closest phylogenetically neighbours were below 63.0%. The peptidoglycan amino acids were alanine, valine, glutamic acid, glycine, ornithine, lysine and aspartic acid. Strain THG-AG1.5T contained ribose, mannose and glucose as whole-cell-wall sugars and menaquinone-8 (MK-8) as the only isoprenoid quinone. The major component in the polyamine pattern was spermidine. The major polar lipids of strain THG-AG1.5T were a phosphoglycolipid, six unidentified glycolipids and an unidentified aminophospholipid. The major fatty acids were identified as iso-C₁₅ : 0, C₁₅ : 1ω₆c, C₁₆ : 0, iso-C₁₇ : 0, C₁₇ : 0, C₁₈ : 0 and summed feature 3. On the basis of our polyphasic taxonomy study, strain THG-AG1.5T represents a novel species within the genus Deinococcus, for which the name Deinococcus hibisci sp. nov. is proposed. The type strain is THG-AG1.5T (=KACC 18850T =CCTCC AB 2016078T).

The genus Deinococcus was first described by Brooks and Murray [1], and later emended by Rainey et al. [2]. The genus Deinococcus is the type genus of the family Deinococaceae in the phylum ‘Deinococcus-Thermus’ [3]. At the time of writing, the genus Deinococcus comprises 59 species with validly published names (www.bacterio.net/deinococcus.html). Some members of the genus Deinococcus show significant resistance to radiation and other sources of oxidative damage by repairing their DNA with unparalleled efficiency compared to other known bacterial species [3, 4]. The bacterial species of this genus have been isolated from various sources, including hot springs [5], water [6, 7], soil [8, 9], desert soil [10–12], radioactive sites [13, 14] and rhizosphere of Ficus religiosa [15]. Members of the genus Deinococcus are characterized as spherical- or rod-shaped, non-motile, aerobic and catalase-positive. These bacteria are generally Gram-stain-positive, but some strains are Gram-stain-negative. The peptidoglycan is type Aβ3 and contains L-ornithine, but no detectable teichoic acids. Menaquinone 8 (MK-8) is the predominant respiratory quinone. They are chemoorganotrophic and their metabolism is respiratory. Their phospholipids do not include phosphatidylglycerol, diphosphatidylglycerol or derivatives. Fatty acids are saturated or monounsaturated. Unbranched 15, 16, and 17 carbon acids predominate in mesophilic species, and branched 16 and 17 carbon acids in thermophilic species. In this study, we report on the taxonomic characterization of a novel species, Deinococcus hibisci sp. nov. by using a polyphasic approach.

Strain THG-AG1.5T was isolated from rhizosphere of a mugunghwa flower plant (Hibiscus syriacus L.), collected in Kyung Hee University (37° 16’ 33” N 127° 10’40” E),

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Keywords: Deinococcus hibisci; Hibiscus syriacus; mugunghwa flower; phenotypic analysis.

Abbreviations: CMC, carboxymethyl cellulose; 2D-TLC, 2-dimensional thin layer chromatography; LA, Luria-Bertani agar; MA, marine agar; MCA, MacConkey agar; MIDI, Sherlock Microbial Identification system; MK-8, menaquinone-8; NA, nutrient agar; R2A, rea soner’s 2A agar; TSA, trypticase soy agar; TSB, trypticase soy broth.

The GenBank accession number for the 16S rRNA gene sequence of strain THG-AG1.5T is KX263319.

Five supplementary figures and one supplementary table are available with the online version of this article.
Yongin, Gyeonggi, Republic of Korea. Sampling was conducted in the area comprising the root region of wild mugunghwa flowers grown in a wooded zone surrounding the university.

One gram of the root material was suspended in 10 ml sterile water containing 0.85% (w/v) NaCl. Serial dilutions were prepared up to $10^{-5}$ using the same solution. Subsequently, 100 ml of each diluted sample was plated onto nutrient agar plates (NA; Difco). After 2 days of culturing at 28 °C, isolates were picked up and purified. For long-term storage, isolates were preserved in nutrient broth (NB; Difco) supplemented with glycerol (25%, w/v) at −80°C.

Extraction of the genomic DNA was achieved by using a commercial genomic DNA extraction kit (Biofact). The 16S rRNA gene was amplified from the chromosomal DNA with the universal bacterial primer pairs, 27F-1492R and 518F-805R according to Frank et al., with modifications [16] and the purified PCR products were sequenced by Biofact (Republic of Korea). The 16S rRNA gene sequences of related taxa were obtained from the GenBank database [17]. Multiple alignments were performed by using the CLUSTAL_X program [18]. Gaps were edited in the BioEdit program [19] and evolutionary distances were calculated using the Kimura two-parameter model [20]. The phylogenetic trees were reconstructed using the neighbour-joining method [21] and the maximum-likelihood method in the MEGA5 program [22], with bootstrap values based on 1000 replications [23].

The 16S rRNA gene sequence of strain THG-AG1.5T determined in this study is a continuous stretch of 1425 bp. According to the EzTaxon-e server (https://www.ezbiocloud.net) [17], multiple alignments were performed by using the CLUSTAL_X program [20]. Gaps were edited in the BioEdit program [19] and evolutionary distances were calculated using the Kimura two-parameter model [20]. The phylogenetic trees were reconstructed using the neighbour-joining method [21] and the maximum-likelihood method in the MEGA5 program [22], with bootstrap values based on 1000 replications [23].

The 16S rRNA gene sequence of strain THG-AG1.5T determined in this study is a continuous stretch of 1425 bp. According to the EzTaxon-e server, strain THG-AG1.5T exhibited high 16S rRNA gene sequence similarity to Deinococcus daejeonensis MJ27T (98.03%), Deinococcus radiotolerans C1T (97.61%) and Deinococcus grandis DSM 3963T (97.32%). Lower sequence similarity (<97%) was found with all other species with valid names of the genus Deinococcus. The relationship between strain THG-AG1.5T and other members of the genus Deinococcus was also supported by the phylogenetic trees topology (Figs 1 and S1 available in the online version of this article).

Gram-staining was determined using a bioMérieux Gram-stain kit according to the manufacturer’s instructions. The growth of strain THG-AG1.5T was tested on several bacterial culture media including NA, tryptase soy agar (TSA; Oxoid), R2A agar, Luria–Bertani agar (LA; Oxoid), marine agar (MA; Difco) and MacConkey agar (Oxoid) at 28 °C. Growth at different temperatures (4, 10, 15, 20, 25, 30, 35, 38 and 42 °C) and at various pH conditions (pH 5.0–10.0, at intervals of 0.5 pH units) were tested in tryptase soy broth (TSB) after 5 days of incubation at 28 °C. For the pH experiments, two different buffers were used (final concentration, 100 mM): acetate buffer was used for pH 5.0–6.5 and phosphate buffer was used for pH 7.0–10.0. Salt tolerance was tested in TSB supplemented with 0–5.0% (w/v) additional NaCl (at 0.5% intervals) after 5 days of incubation at 28 °C. Growth was estimated by monitoring the optical density at 600 nm. Anaerobic growth was tested in serum bottles containing TSB supplemented with thioglycolate (1 g l$^{-1}$), in which the air was substituted with nitrogen gas. Cell morphology was observed at ×11000 magnification, with a transmission electron microscope (Model JEM1010; JEOL) using cells grown for 2 days at 28 °C on TSA. Motility was assayed in sulfide–indole motility medium (SIM; Difco). Production of flexirubin-type pigments was determined by following the procedures outlined by Fautz and Reichenbach [24]. Catalase activity was determined by bubble production in 3% (v/v) H$_2$O$_2$ and oxidase activity was determined using 1% (w/v) N,N,N',N''-tetramethyl-1,4-phenylenediamine reagent. Tests for degradation of starch [1% (w/v), Difco], casein [2% (w/v) skim milk, Oxoid], DNA (DNase agar, Oxoid), Tween 80 [1% (w/v), Sigma], L-tyrosine [0.5% (w/v), Sigma], CMC [0.1% (w/v) CM-cellulose, Sigma] and chitin from crab shells [1.0% (w/v), Sigma] were evaluated after 5 days of incubation at 28 °C. The strains D. daejeonensis MJ27T, D. radiotolerans and D. grandis DSM 3963T were included as references for the investigation of the biochemical tests using the same laboratory conditions. Carbon-source utilization and constitutive enzyme activities of strain THG-AG1.5T and the three references strains above were tested by using API 20NE, API 32 GN and API ZYM test kits according to the instructions of the manufacturer (bioMérieux). In addition, acid production from different carbohydrates was determined for novel strain THG-AG1.5T and its closest reference strains by employing the API 50CH system according to the instructions of the manufacturer (bioMérieux). The API kits were incubated at 28 °C, and the results were obtained after 24–48 h.

Cells of strain THG-AG1.5T were Gram-stain-positive, coccus-shaped, non-motile and exhibited aerobic growth. The cell size was approximately 2.7–1.9×1.3–0.9 μm (Fig. S2). Oxidase- and catalase-activities were positive. Flexirubin-type pigments were not produced. Colonies grown on TSA plates for 3–4 days were smooth, bright, flat, circular, pink-coloured and 0.5–0.8 mm in diameter. The isolate grew well on R2A, TSA and NA, grew weakly on LA and MacConkey agar, but not at all on MA. The temperature range for growth was 15–38 °C (optimum 25–30 °C) and at pH 6.0–8.5 (optimum 6.5–7.5). The strain had an optimum growth in the presence of additional 1.5% (w/v) NaCl, but it tolerated up to 2.5% (w/v) NaCl. Cells were able to hydrolyse Tween 80 and CMC but not L-tyrosine, casein, chitin, starch and DNA. Other physiological and biochemical characteristics of strain THG-AG1.5T are summarized in the species description, and a comparison of selected characteristics of strain THG-AG1.5T and related strains is given in Table 1.

To determine the tolerance of strain THG-AG1.5T to UV and gamma radiation, cells were grown at 28 °C in tryptone glucose yeast broth (TGY broth) to the exponential phase ($=10^9$ c.f.u. ml$^{-1}$). D. daejeonensis MJ27T, D. radiotolerans
and *D. grandis* DSM 3963<sup>T</sup> were tested as positive controls and *Escherichia coli* K12 (=KCTC 1116) as negative control. To determine the survival fraction after exposure to UV radiation, cultured cells were serially diluted with sterile PBS; 0.1 ml aliquots from each dilution were spread on TSA plates and were exposed (with the lids open) to UV light (UVP CX-2000; 254 nm) to dosages of 200, 400, 600, 800, 1000 and 1500 J m<sup>-2</sup>. Irradiated cells on the agar plates were then incubated in the dark at 28°C for 48 h. To measure ionizing radiation resistance, cultures grown to the early stationary phase (≈10<sup>9</sup> c.f.u. ml<sup>-1</sup>) were serially diluted with sterile PBS and later divided into 0.1 ml aliquots, on ice, to a cobalt-60 gamma irradiator (AECL, IR-79; Korea Atomic Energy Research Institute). The source strength was approximately 100 kCi at a dose rate of 70 Gy min<sup>-1</sup>; actual doses were within 2 % of the target dose. Irradiated cells were diluted, plated on TSA plates and incubated at 28°C for 48 h, after which survivors were scored.

Strains THG-AG1.5<sup>T</sup>, *D. daejeonensis MJ27<sup>T</sup>*, *D. radiotolerans* and *D. grandis* DSM 3963<sup>T</sup> could grow from the irradiated cells at all dosages. No growth of colonies of *E. coli* K12 (=KCTC 1116) was observed on plates irradiated at 200 J m<sup>-2</sup> or higher dosages of UV radiation. The novel strain THG-AG1.5<sup>T</sup> exhibited higher UV resistance, evaluated by D<sub>10</sub> values (≥1500 J m<sup>-2</sup>), than its closest reference strains (Fig. S4). After examining the survival fraction after exposure to gamma radiation, strain THG-AG1.5<sup>T</sup> and its closest reference strains, *D. daejeonensis MJ27<sup>T</sup>*, *D. radiotolerans* and *D. grandis* DSM 3963<sup>T</sup>, showed resistance to a D<sub>10</sub> value in excess of 12 kGy. The negative control, *E. coli* K12 (=KCTC 1116), was sensitive to a D<sub>10</sub> value of 3 kGy (Fig. S5).

For determination of the DNA G+C content, genomic DNA was extracted, purified as described by Moore and Dowhan [25], and degraded enzymatically into nucleosides. The nucleosides were analysed using a reverse-phase
and
leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin,
positive for alkaline phosphatase, esterase (C4), esterase lipase (C8),
all strains are negative for
late, 3-hydroxy-butyrate and
strains are positive for
PNPG,
hydrolysis of aesculin and gelatin. In API 20NE tests, all species are
positive for PNPG, glucose and d-mannitol; negative for indole pro-
duction, caprate, adipate and phenyl-acetate. In API 32 GN tests, all
strains are positive for d-sorbitol, l-proline, sucrose and glycogen;
negative for l-fucose, propionate, 4-hydroxy-benzoate, inositol, suber-
ate, 3-hydroxy-butylate and l-serine. In API ZYM tests, all strains are
positive for alkaline phosphatase, esterase (C4), esterase lipase (C8),
leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin,
naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase
and β-glucosidase; weakly for lipase (C14); negative for N-acetyl-β-
glucosaminidase, α-mannosidase and α-fucosidase. In API 50CH tests,
all strains are negative for d-xylene, l-xylene, raffinose and l-fucose.
+, Positive; w, weakly positive; —, negative.

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<td>Melibiose</td>
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<td>Sodium acetate</td>
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<td>D,L-Lactate</td>
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Table 1. Physiological characteristics of strain THG-AG1.5T and related type strains

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<td>Amygdalin</td>
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<td>Salicin</td>
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<td>Lactose</td>
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<td>Trehalose</td>
<td>w</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>74.8</td>
<td>67.6</td>
<td>68.59</td>
<td>68.4</td>
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The DNA G+C content of strain THG-AG1.5T was 74.8 mol %, a value above the range reported for species of the genus Deinococcus [28]. The DNA–DNA relatedness values for strain THG-AG1.5T with respect to D. daejeonensis MJ27T, D. radiotolerans C1T and D. grandis DSM 3963T were 63.0 ±0.7, 51.6±1.0 and 48.2±0.3, respectively. All DNA–DNA relatedness values were lower than the threshold value of 70 % recommended for recognition of separate species [29].

Freeze-dried cells for the analysis of polar lipids, quinones, cell sugars and peptidoglycans were produced from cultures grown for 2–3 days in TSB under aerobic conditions at 28 °C with shaking (180 r.p.m.). Isoprenoid quinones of strain THG-AG1.5T were extracted from freeze-dried cell material. Menaquinones were extracted with chloroform/ methanol (2:1, v/v), concentrated using a rotatory evaporator at 50 °C and extracted in only n-hexane. The crude hex-
ane/quinone solution was purified using Sep-Pak Vac silica

Table 1. cont.
methanol/water (65:25:4, v/v/v) and (2) chloroform/acetic acid/methanol/water (80:15:12:4, v/v/v/v) as the developing solvents. After being developed in the solvent system, lipids were visualized. To detect the presence of lipids, TLC plates were developed by spraying with 5% molybdenum phosphoric acid and charred at 120 °C for 10 min. Aminolipids were detected by spraying with 0.2% ninhydrin at 120 °C for 5 min. Phospholipids were detected by spraying with molybdenum blue reagent (Sigma) at room temperature. Glycolipids were visualized with 2.5% α-naphthol-sulfuric acid by charing at 120 °C for 5 min. Peptidoglycan and whole-cell sugars of strain THG-AG1.5T and D. dajeo-

nensis MJ27T were determined by using cellulose plates for TLC as described by Schleifer and Kandler [36], and Stan
cek and Roberts [37], respectively. For fatty acid methyl ester analysis, cells of strain THG-AG1.5T and type strain were harvested in TSA after incubation for 2 days at 28 °C and fatty acids determined as described by Sasser [38]. The polyamines of strain THG-AG1.5T were extracted as described by Busse and Auling [39], and Taibi et al. [40]. Polyamines were extracted from approximately 100 mg freeze-dried samples with 2 ml 0.2 N HClO4. Experiments were designed to be carried out at 100 °C for 30 min with occasional shaking. Each mixture contained 1.8-diaminoch
tane (10 μmol/100 mg of cells) as the internal standard. Samples were analysed using Waters Alliance 2690 HPLC system fitted with a reverse-phase column (Watchers 120 ODS-AP 4.6×250 mm×5 μm) at 1.0 ml min⁻¹ flow rate, wavelength at 234 nm and 60% methanol as mobile phase.

The major respiratory quinone of strain THG-AG1.5T was identified as menaquinone-8 (MK-8), in line with all other members of the genus Deinococcus. The polar lipid profile of strain THG-AG1.5T is shown in Fig. S3. The polar lipid profile of the novel species included six unidentified glyco-
lipids, a phosphoglycolipid, an unidentified aminophospholipid, an unidentified lipid and two unidentified aminolipids. The predominant polar lipid of strain THG-AG1.5T was a phosphoglycolipid, identified in D. radu

durans as 2'-O-(1,2-diacyl-sn-glycero-3-phospho)-3'-O-
(α-galactosyl)-N-d-glyceroyl alkylamine [41], which is common to members of the genus Deinococcus [42, 43]. Moreover, the presence of various unidentified glycolipids [11, 33] and an unidentified aminophospholipid related THG-AG1.5T with other deinococci [15, 44, 45]. However, this novel strain was readily distinguished from other Deinococcus species by the presence of an unidentified lipid and two unidentified aminolipids. The major peptidoglycan amino acids contained in strain THG-AG1.5T were alanine, valine, glutamic acid, glycine, ornithine, lysine and aspartic acid. The inter-peptide bridge of the cell-wall peptidoglycan of strain THG-AG1.5T comprised ornithine and glycine as diagnostic amino acids, corresponding to peptidoglycan structure type A3β, which is characteristic of the genus Deinococcus [3]. The whole-cell sugars found in strain THG-AG1.5T and the closest reference strain were ribose, mannose and glucose. The fatty acid profiles of strain THG-AG1.5T and the related Deinococcus type

strains are shown in Table S1. The major cellular fatty acids were identified as iso-C15:0 (9.8%), C15:1ω6c (11.8%), C16:0 (15.2%), iso-C17:0 (14.3%), C17:0 (8.1%), C18:0 (7.1%) and summed feature 3 (C16:1ω7c and/or C16:1ω6c; 10.5%). The major polyamine was spermidine, characteristic of the polyamine pattern of species within the genus Deinococcus [46].

In summary, the characteristics of strain THG-AG1.5T were consistent with descriptions of the genus Deinococcus with regard to morphological, biochemical and chemotaxonomic properties. The results of this polyphasic approach between strain THG-AG1.5T and its closest phylogenetic neighbours indicated that strain THG-AG1.5T should be assigned to the genus Deinococcus as a novel species, for which the name Deinococcus hibisci sp. nov. is proposed.

**DESCRIPTION OF DEINOCOCCUS HIBISCI SP. NOV.**

Deinococcus hibisci (hi.bi'sči. L. gen. n. hibisci of the plant genus Hibiscus).

Cells are Gram-positive cocci, 2.7–19×1.3–0.9 μm, strictly aerobic and non-motile. Colonies are smooth, bright, flat, circular, pink-coloured and 0.5–0.8 mm in diameter. Catalase and oxidase activities are positive. Flexirubin-type pigments are not produced. Can grow in TSB at 15–38 °C, optimum growth occurs from 25–30 °C. Growth occurs on R2A, TSA and NA agar, grows weakly on LA and MacCon
dkey agar but not on MA. Strain THG-AG1.5T can grow in TSB at pH 6.0–8.5 (optimum 6.5–7.5) and in the presence of additional 0–1.5% (w/v) NaCl. Cells are able to hydrolyse Tween 80 and CMC but not l-tyrosine, casein, chitin, starch and DNA. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosi
dase, α-glucosidase and β-glucosidase; weakly positive for lipase (C14); negative for α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosi
dase. Positive for reduction of nitrate to nitrites, assimilation of PNPG, arginine dihydrolase, D-glucose, L-arabinose, D-mannose, D-mannitol, maltose, and hydrolysis of urea, aesculin and gelatin; weakly positive for trisodium citrate; negative for indole production, glucose acidification, assimilation of N-acetyl-glucosamine, gluconate, caprate, adipate, malate and phenyl-acetate. Positive for utilization of salicin, melibiose, D-sorbitol, L-histidine, 2-ketogluconate, 3-
dhydroxy-butyrate, L-proline, sucrose, l-alanine and glyco
gen; weakly positive for valerate, D-ribose, itaconate, sodium malonate, sodium acetate and D,L-lactate; negative for L-fucose, propionate, 4-hydroxy-benzoate, L-rhamnose, inositol, suberate, 5-ketogluconate, 3-hydroxy-benzoate and L-serine. Weakly positive for assimilation of glycerol, D-fruc
tose and trehalose; negative for erythritol, D-arabinose, D-xylene, L-xylene, D-adonitol, methyl β-D-xylpyranoside, D-galactose, L-sorbosé, dulcitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetyl-D-glucosamine,
amylodalin, arbutin, cellobiose, lactose, inulin, melezitose, raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, D-arabitol and L-arabitol. The predominant respiratory quinone is menaquinone-8 (MK-8); and iso-C_{15:0}, C_{15:1ω6c}, C_{16:0}, iso-C_{17:0}, C_{17:0}, C_{18:0} and summed feature 3 (C_{16:1ω7c} and/or C_{16:1ω6c}) are the major components of the cellular fatty acids (≥7%). The major polar lipids are a phosphoglycolipid, six unidentified glycolipids and an unidentified aminophospholipid. The polyamine is spermidine. The novel strain exhibits tolerance to UV irradiation (>1500 J m⁻²) and to gamma radiation (≥12 kGy). The G+C content of genomic DNA of strain THG-AG1.5 is 74.8 mol%. The peptidoglycan amino acids are alanine, valine, glutamic acid, glycine, ornithine, lysine and aspartic acid. The whole-cell-wall sugars are ribose, mannose and glucose.

Strain THG-AG1.5 (KACC 18850=CCTCC AB 2016078) was isolated from a rhizosphere soil sample of Hibiscus syriacus L. (Mugunghwa flower), collected in Kyung Hee University, Yongin, Gyeonggi, Republic of Korea.

Funding information
This work was conducted under the industrial infrastructure program for fundamental technologies which is funded by the Ministry of Trade, Industry and Energy (MOTIE), Korea (no. N0000888).

Conflicts of interest
The authors declare that there are no conflicts of interest.

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