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Hymenobacter terrae sp. nov., a Bacterium Isolated from Soil

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Abstract A Gram-negative, UV tolerant bacterial strain, DG7A^T, was isolated from soil samples collected in Seoul city, South Korea. The cells were grown on R2A agar at 25 °C and were pink to red in color. The DNA G+C content of the novel strain DG7A was 63.5 mol%. Chemotaxonomic data revealed that the strains contain the major fatty acids iso-$C_{15:0}$, anteiso-$C_{15:0}$, and summed feature 3 (16:1 ω7*c*/16:1 ω6*c*), with phosphatidylethanolamine as the major polar lipid. Phylogenetic analysis of the 16S rRNA gene sequences showed that strain DG7A^T formed a distinct phylogenetic line along with *Hymenobacter soli* PB17^T, and they shared approximately 98.35 % 16S rRNA gene sequence similarity. However, these two strains shared only 5.3 % pairwise similarity (reciprocal analysis, 36.3 %) in their genomic DNA. The next highest degree of 16S rRNA gene sequence similarity after *H. soli* PB17^T was found with *H. glaciei* VUG-A130^T (96.78 %), *H. antarcticus* VUG-A42aa^T (96.66 %), and *H. saemangeumensis* GSR0100^T (96.57 %). Based on the phylogenetic analysis and analysis of the physiological and biochemical characteristics, this isolate was considered to represent a novel species, for which we propose the name *Hymenobacter terrae* sp. nov., with type strain DG7A^T (= KCTC 32554^T = KEMB 9004-164^T = JCM 30007^T).

The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DG7A^T (= KCTC 32554^T = KEMB 9004-164^T = JCM 30007^T) is KF862488.

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Introduction

The genus *Hymenobacter* was proposed as a novel genus by Hirsch et al. [9]. The genus *Hymenobacter* belongs to the family *Cytophagaceae*, the order *Sphingbacteriales*, and the phylum *Bacteroidetes* and is well known for having higher DNA G+C contents (55–70 mol%) compared with other genera of the family. *Hymenobacter* species are pink to red pigmented, rod-shaped, non-motile, and Gram-negative bacteria that contain phosphatidylethanolamine (PE) as the main phospholipid, menaquinone 7 as the major quinone, and iso-$C_{15:0}$, anteiso-$C_{15:0}$, and summed feature 3 (16:1 ω7*c*/16:1 ω6*c*) as the major fatty acids.

At the time of writing, the genus was comprised of 28 recognized species, which were discovered in various environments. Overall, many species of the genus have been isolated from soil samples. These include the following: *Hymenobacter arizonensis,* isolated from the southwestern arid lands soil [22]; *H. daecheongensis,* from stream sediment [37]; *H. deserti* and *H. xinjiangensis,* from the desert soil of Xiinjian, China [39, 40]; *H. ginsengisoli,* from the soil of a ginseng field [10]; *H. psychrophilus,* from the soil of an industrial site [38]; and *H. ruber* and *H. soli,* from grass soil [12, 14].

In this study, strain DG7A^T was isolated from a soil sample. Based on a polyphasic approach including phenotypic, phylogenetic, genomic, and chemotaxonomic

characteristics, strain DG7A^T was designated as a new species in the genus *Hymenobacter*.

Materials and Methods

Isolation of Bacterial Strain and Culture Conditions

Strain DG7A^T was isolated from a soil sample (1.0 g) collected in Seoul (N 37° 34′ 30″ E 127° 00′ 30″), South Korea. The soil sample was suspended in 10.0 ml sterile water, after which the resulting supernatant was serially diluted. 100 µl of each dilution was spread on a plate of R2A agar (Difco, USA) which had been diluted 10 times, and was incubated at 25 °C. The purified colonies were tentatively identified by partial 16S rRNA gene sequences, and were preserved in a glycerol solution (25 %, w/v) at −70 °C. Strain DG7A^T was deposited into the Korean Collection for Type Cultures (KCTC 32554^T) and the Japan Collection of Microorganisms (JCM 30007^T).

Phenotypic and Biochemical Characteristics

The Gram reaction was performed using the non-staining method [2]. Cell morphology was observed using a light microscope (Nikon, Japan) and a transmission electron microscope (LIBRA 120, Carl Zeiss, Germany). Oxidase activity was evaluated via the oxidation of 1 % (w/v) tetramethyl-*p*-phenylene diamine. Catalase activity was determined by measurement of bubble production after the application of a 3 % (v/v) H_2O_2 solution. Growth at various pHs (5–11 at pH 1 intervals) was assessed on R2A broth at 25 °C. To determine the NaCl tolerance, R2A agar containing different salt concentrations [0–10 % (w/v %), 1 % intervals] were used. Growth on different media was also assessed, sing tryptic soy agar (TSA; Difco), nutrient agar (NA; Difco), Luria–Bertani (LB; Difco) agar, and *Ancylobacter–Spirosoma* Medium (ASM; glucose 1 g, peptone 1 g, yeast extract 1 g, agar 15 g). All of the above growth tests were performed at 25 °C. Growth at different temperatures (4, 12, 17, 20, 25, 30, 37, and 42 °C) was assessed on R2A agar plates for 3–7 days. The API 20NE, API 50CH, and API ZYM microtest systems were employed according to the manufacturer's instructions (bioMérieux), for studying carbon source utilization and the enzyme activities of the strains.

Pigments were extracted using 95 % ethanol, and the absorption spectrum between 200 and 800 nm was measured with a UV spectrophotometer (UV-2450, Shimazu). Flexirubin-type pigments were tested for based on a color shift after exposure to 0.1 N NaOH solution [7, 35].

Survival Assays to Ultraviolet

Cells were grown up to early stationary phase on R2A broth (Difco) and adjusted to $\sim 10^8$ CFU/m [11, 27]. Cells were then serially diluted with 0.85 % NaCl and spotted on R2A agar. After taking off the lids, cells on the R2A agar plates were exposed to UV at room temperature using a UVC ultraviolet crosslinker (UVP, CX-2000, CA, USA) at 254 nm. The applied dose rate was 20 J/m^2/s, and different doses of radiation were achieved by adjusting the total exposure time. After exposure, cells were incubated at 30 °C for 2 days prior to enumeration of the colonies.

16S rRNA Gene Sequencing and Phylogenetic Analysis

The 16S rRNA gene of strain DG7A^T was amplified from chromosomal DNA using the universal bacterial primer set, 9F, and 1492R [36]. Sequence analysis was performed using the 27F, 785F, 805R, and 1492R universal primers from Genotech (Daejeon, South Korea). The full sequences of the 16S rRNA gene were compiled with SeqMan software (DNASTAR Inc.).

For phylogenetic analysis, the nearly full sequence of strain DG7A^T obtained (1461 bp) was compared with other taxa using the EzTaxon-e service [15]. The 16S rRNA gene sequences of related taxa were obtained from GenBank and were edited with the BioEdit program [8]. Multiple alignments were performed with the CLUSTAL X program [32]. Pairwise distances for the neighbor-joining algorithm [24] were calculated according to Kimura two-parameter model [16], and the phylogenetic tree was constructed using the MEGA 5 Program [31]. Bootstrap analysis with 1,000 replicates was also conducted to obtain confidence levels for the branches [6]. The close-neighbor-interchange (CNI) on random trees method with a search factor of one and the number of initial trees (random addition) of ten was applied in maximum parsimony analysis, and maximum likelihood analysis was performed with the kimura 2-parameter model in the MEGA 5 Program.

Chemotaxonomic Analyses

To analyze the composition of polar lipids, total lipids were extracted following the procedures of Minnikin et al. [21]. The total lipids were separated using two-dimensional thin layer chromatography (TLC) with the first and second developing solvents, chloroform/methanol/water (65:25:4), and chloroform/methanol/acetic acid/water (80:12:15:4), respectively. After separation of the lipids, various lipids were tested for by spraying with the appropriate detection reagents [19]. The total lipids were verified by heating for 10 min at 150 °C after spraying with a molybdophosphoric acid solution (Sigma-Aldrich, St. Louis, MO, USA). In

order to perform fatty acid methyl ester analysis, cells were allowed to grow on R2A agar for 3 days at 25 °C, and then two loops of well-grown cells were harvested. Fatty acids were purified by saponification, methylation, and extraction procedures, as described previously [25]. Fatty acid methyl esters (FAME) were prepared, separated, and identified with the Sherlock Microbial Identification System V6.01 (MIS, data base TSBA6, MIDI Inc., Newark, DE, USA).

Genomic Analysis

DNA–DNA hybridization was performed fluorometrically, according to the method reported by Ezaki et al. [5]. For the determination of the G+C mol content of strain DG7A^T and relative taxa, genomic DNA was extracted using the standard method, which employs a CTAB/NaCl solution. The purified genomic DNA was enzymatically degraded into nucleosides by nuclease P1 and alkaline phosphatase, and the nucleosides were then analyzed using reverse-phase HPLC as previously described in the literature [20, 30].

Results and Discussion

Morphological and Phenotypic Characteristics

Cells of strain DG7A^T were Gram-negative, non-motile, and rod-shaped without flagella (Supplementary Fig. S1), exhibiting a red-pink color when cultured on R2A agar at 25 °C. The red-pink pigment produced by strain DG7A^T had the spectrum of a carotenoid pigment with absorption maxima at 264.5, 319.0, 368.0, and 480.0 (pentosyl-2′-hydroxyflexixanthin [17]) nm (Supplementary Fig. S2). This result differs from that of *H. saemangeumensis* GSR0100^T (453.0, 483.0 (hexosyl-2′-hydroxyflexixanthin or methyl-2′-hydroxyflexixanthin [17]), and 508.0 nm) [13]. Alkalization with 0.1 volume of 0.1 M NaOH did not lead to any shift in peak positions, indicating that strain DG7A^T did not have flexirubin pigment. Cells were able to grow at temperatures between 12 and 30 °C with an optimum of 25 °C but did not grow at 4, 37, and 42 °C. The optimum pH of strain DG7A^T was found to be between 6.0 and 9.0, and weak growth was observed at pH 5 and pH 10–11. Strain DG7A^T did not grow in the examined NaCl (w/v) range (0.5–10 %). Results regarding the physiological characteristics of strain DG7A^T were summarized in the species description, and the differential characteristics with type strains of closely related species are shown in Table 1.

Phylogenetic Analysis

16S rRNA gene (1461 bp) sequence of strain DG7A^T was compared with those of closely related taxa. The phylogenetic tree constructed by the neighbor-joining method revealed that strain DG7A^T belongs to the genus *Hymenobacter* (Fig. 1). Strain DG7A^T had the highest sequence similarity with *Hymenobacter soli* PB17^T (98.35 %) [14], *H. glaciei* VUG-A130^T (96.78 %) [18], *H. antarcticus* VUG-A42aa^T (96.66 %) [18], and *H. saemangeumensis* GSR0100^T (96.57 %) [13]. Levels of sequence similarity to other genera (*Adhaeribacter* and *Nibribacter*) were less than 89.32 %.

Chemotaxonomic Characteristics

The major cellular fatty acids of strain DG7A^T were iso-$C_{15:0}$ (24.87 %), anteiso-$C_{15:0}$ (21.42 %), and summed feature 3 ($C_{16:1}$ $\omega6c$ and/or $C_{16:1}$ $\omega7c$) (15.01 %), which are predominant in most *Hymenobacter* species. The minor fatty acids of strain DG7A^T were iso-$C_{14:0}$, iso-$C_{15:0}$ 3OH, iso-$C_{15:1}$ G, $C_{16:0}$, iso-$C_{16:0}$, iso-$C_{16:0}$ 3OH, iso-$C_{16:1}$ H, $C_{16:1}$ $\omega5c$, iso-$C_{17:0}$, antesio-$C_{17:0}$, $C_{17:0}$ 2OH, iso-$C_{17:0}$ 3OH, and Summed Feature 5 (anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I). The major cellular fatty acids of strain DG7A^T are common to closely related species. However, some qualitative and quantitative differences in the fatty acid composition were observed between the novel strain and the other closely related *Hymenobacter* species. Strain DG7A^T had smaller amounts of iso-$C_{15:1}$ G (1.64 %), whereas other *Hymenobacter* species (*H. soli* KACC 13040^T and *H. glaciei* JCM 17225^T) had larger amounts of the corresponding fatty acids. In addition, the fatty acids iso-$C_{14:0}$ and anteiso-$C_{17:0}$ composed of more than 1 % of the fatty acids in strain DG7A^T, but were present at less than 1 % or not detected at all in the closely related species. Strain DG7A^T also had larger amounts of $C_{16:0}$ (5.31 %), whereas other closely related *Hymenobacter* species had smaller amounts of the corresponding fatty acids (Table 2). The major polar lipid found in strain DG7A^T was phosphatidylethanolamine (PE), which is similar to other *Hymenobacter* species [3, 13]. Minor amounts of an unknown aminolipid (AL), unknown aminophospholipid (APL), unknown glycolipid (GL), and unknown polar lipid (L) were also found (Supplementary Fig. S3).

Genomic Characteristics

When examining DNA, strain DG7A^T exhibited low DNA–DNA relatedness values with the closely related species *H. soli* KACC13040^T (5.3 ± 1.8 %; reciprocal analysis, 36.3 ± 2.2 %), *H. glaciei* JCM17225^T (5.6 ± 0.8 %), *H. antarcticus* JCM17213^T (3.2 ± 0.8 %), and *H. saemangeumensis* KACC16452^T (8.1 ± 0.8 %) (Supplementary Table 1). DNA–DNA hybridization levels between strain DG7A^T and other type strains were determined to be

Table 1 Differential characteristics between strain DG7A^T and closely related species

	1	2	3	4	5
Gram reaction (3 % KOH)	Negative	Negative	Negative	Negative	Negative
Size (μm long) (by TEM)	2.1–3.0	2.0–4.1[a]	1.0–2.0[a]	1.0–2.0[a]	1.4–3.6[a]
Size (μm wide) (by TEM)	0.6–1.1	0.6–1.1[a]	0.5–0.8[a]	0.5–1.0[a]	0.5–0.8[a]
Nitrate reduction to nitrite	−	−	−	+	−
Production of acid from glucose	−	−	−	+	−
Growth-permissive temperature (°C)[a]	12–30	4–30[a]	4–28[a]	4–18[a]	15–30[a]
Growth-permissive pH range[a]	5–11	5–8.5[a]	5–11[a]	6–11[a]	5.5–10[a]
Enzyme activity					
Catalase	+	+	+	−	+
N-Acetyl-*β*-glucosaminidase	+	w	w	+	+
Arginine dihydrolase	−	−	+	−	−
α-Chymotrypsin	−	−	w	−	−
α-Galactosidase	+	−	−	−	−
β-Galactosidase (ONPG)	+	+	−	−	−
β-Galactosidase (PNPG)	+	w	−	−	−
α-Glucosidase	w	−	w	−	−
β-Glucosidase (esculin hydrolysis)	+	+	+	+	−
Protease (gelatin hydrolysis)	+	−	−	+	+
Trypsin	−	−	−	w	−
Urease	−	−	+	−	−
Fermentation					
L-arabinose	w	w	−	−	−
Esculin	w	+	+	w	−
D-fructose	w	w	−	−	−
D-galactose	w	−−	−	−	w
Glycogen	w	−	−	w	+
D-lactose	w	+	−	−	−
Maltose	w	+	−	+	+
D-mannose	−	−	−	+	w
D-raffinose	w	w	−	−	−
Starch	−	−	−	+	+
D-sucrose	w	+	−	−	−
D-trehalose	w	−	−	−	−
D-xylose	w	−	−	−	−
2-Ketogluconate	−	−	−	w	−
5-Ketogluconate	+	+	+	+	w
Xylitol	−	w	−	−	−
G+C content[a]	63.5 %	57.1 %	NR	NR	61.9 %[a]

All strains are Gram negative and oxidase positive

All data were obtained in this study, unless otherwise noted

Strains: 1, DG7A^T; 2, *H. soli* KACC13040^T; 3, *H. glaciei* JCM17225^T; 4, *H. antarcticus* JCM17213^T; 5, *H. saemangeumensis* KACC16452^T

(+) positive, (−) negative, *NR* not reported, *w* weakly positive reaction

[a] Data from references [13, 14, 18]

less than 70 %, which is the threshold for delineating a genomic species [28, 34]. Thus, our results support the placement of strain DG7A^T as a representative of a separate and previously unrecognized genomic species.

The G+C contents of genomic DNA from strain DG7A^T and *H. soli* KACC13040^T were 63.5 and 57.1 mol%, respectively (58.8 mol% for *H. soli* PB17^T from Kim et al. [14]). Their G+C contents differ by over 5 %, providing

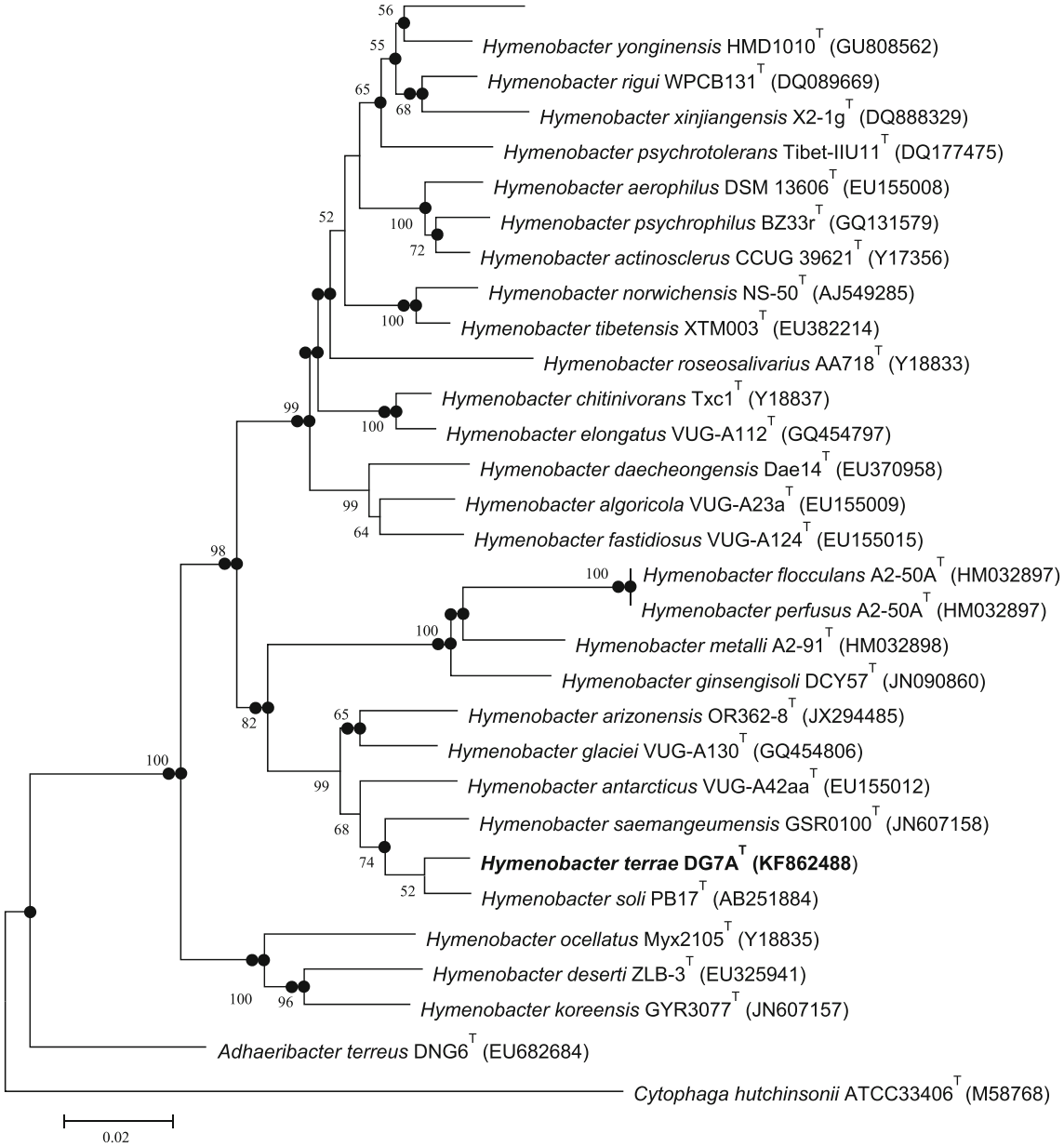

Fig. 1 A neighbor-joining tree based on the 16S rRNA gene sequences showing the phylogenetic relationship between strain DG7A^T and other closely related taxa. A *bar* represents 0.02 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50 % are shown at the branch points. *Filled circles* indicate the common nodes recovered from either the maximum parsimony algorithm or the maximum likelihood tree. *Filled double circles* indicate that the corresponding nodes were recovered in both the maximum parsimony tree and the maximum likelihood tree

additional support that strain DG7A^T is not the same species, but the same genus as *H. soli* KACC13040^T. It is now generally accepted that bacteria with DNA differing by more than 5 % G+C content should not be assigned to the same species, and those differing by more than 10 % should not be classified in the same genus [4, 23, 26, 29, 33].

UV Radiation Resistance Analysis

Survival of strain DG7A^T against UV radiation was examined. Strain DG7A^T showed the characteristic shoulder of resistance in survival curves, as shown by UV radiation-resistant *Deinococcus radiodurans* [1] (Supplementary Fig. 4). Whereas *E. coli* could not survive even at

Table 2 Cellular fatty acid profiles of strain DG7A^T and closely related reference strains

Fatty acids	1	2	3	4	5
Saturated					
13:0 iso	tr	1.14	tr	tr	tr
14:0 iso	2.02	tr	tr	tr	tr
15:0 iso	24.87	26.00	13.76	12.04	23.02
15:0 anteiso	21.42	11.42	19.06	15.54	5.45
15:0 2OH	tr	tr	1.26	tr	tr
15:0 iso 3OH	1.99	3.67	1.86	2.39	2.23
16:0	5.31	3.78	1.03	1.42	1.91
16:0 iso	1.71	tr	2.85	tr	2.65
16:0 iso 3OH	1.03	tr	tr	1.74	tr
16:0 3OH	tr	1.15	tr	1.45	tr
17:0 iso	2.62	1.34	2.79	tr	1.78
17:0 anteiso	1.42	nd	tr	nd	tr
17:0 2OH	1.65	tr	1.81	1.29	tr
17:0 iso 3OH	1.11	2.20	1.48	1.06	3.29
Unsaturated					
15:1 iso G	1.64	10.37	12.05	1.57	5.75
15:1 anteiso A	tr	1.43	4.65	tr	tr
16:1 iso H	1.91	1.19	1.72	3.77	3.98
16:1 $\omega 5c$	6.66	7.11	7.53	13.10	9.00
16:1 $\omega 7c$ alcohol	tr	1.60	tr	4.74	2.55
17:1 $\omega 6c$	tr	tr	tr	tr	1.08
Summed feature 3 (16:1 $\omega 7c$/16:1 $\omega 6c$)[a]	15.01	17.70	18.03	26.79	16.28
Summed feature 5 (17:1 anteiso B/iso I)[a]	3.20	3.84	4.42	5.79	12.78
Summed feature 6 (18:0 anteiso/18:2 $\omega 6, 9c$)[a]	nd	nd	nd	nd	1.47

All strains were grown on R2A at 20 °C for 7 days. Values are percentages, and only fatty acids accounting for more than 1 % in at least one of the strains are indicated. For unsaturated fatty acids, the position of the double bond was located by counting from the methyl Ò(ω) end of the carbon chain. The *cis* isomers were indicated by the suffixes *c*

Strains: 1, DG7A^T; 2, *H. soli* KACC13040^T; 3, *H. glaciei* JCM17225^T; 4, *H. antarcticus* JCM17213^T; 5, *H. saemangeumensis* KACC16452^T

nd not detected, *tr* trace (<1.0 %)

[a] Summed feature contained two fatty acids, which could not be separated by GLC with the MIDI system

the lowest dose tested (300 J/m^2), 60.0 % survival was observed at this dose for strain DG7A^T (87.5 % for *D. radiodurans* R1^T), and 0.3 % of DG7A^T cells were able to survive after exposure to UV doses as high as 600 J/m^2.

Taxonomic Conclusion

Strain DG7A^T has similar characteristics as other *Hymenobacter* species, defined by the presence of phosphatidylethanolamine (PE) as the major polar lipid, and iso-$C_{15:0}$, anteiso-$C_{15:0}$, and summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$) as the abundant fatty acids. The result of DNA–DNA hybridization implied that strain DG7A^T was not the same species as the closest related species, as the DNA–DNA relatedness value was lower than 70 %. The physiological characters differentiating the strain DG7A^T from the other reference species are shown in Table 1.

Description of *Hymenobacter terrae* sp. nov

Hymenobacter terrae (*ter'ra. L. gen. n. terrae of the earth)*

Cells are 0.6–1.1 μm wide and 2.1–0.3.0 μm long, Gram-negative, non-motile, and rod-shaped, when grown on R2A agar (Difco) at 25 °C for 3 days. Colonies on R2A agar are pink to red-colored, circular, smooth and slimy. They are positive for both oxidase and catalase activities. The cells are able to grow at a temperature range of 12–30 °C (optimum 25–30 °C), but not at 4, 37, and 42 °C. Cells grow on R2A and ASM, but not on LB, TSA, and NA media at 25 °C. Efficient growth occurs at pH 6–9, with weak growth occuring at pH 5 and 10–11. Cells grow optimally in the absence of NaCl. Cells cannot reduce nitrate to nitrite or nitrogen. Glucose fermentation and indole production are also negative (API 20NE).

In tests with the API Zym system, cells tested positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase (C4), esterase (C8), α-galactosidase, β-galactosidase (OPNG), α-glucosidase (weakly), leucine arylamidase, naphtol-AS-BI-phosphohydrolase, and valine arylamidase. Cells tested negative for α-chymotrypsin, α-fucosidase, β-glucosidase, β-glucuronidase, lipase (C14), α-mannosidase, and trypsin.

Acid is produced weakly (API 50CH) with the use of L-arabinose, esculin ferric citrate, D-fructose, D-galactose, glycogen, 5-ketogluconate (positive), D-lactose, D-maltose, D-raffinose, D-saccharose (sucrose), D-trehalose, and D-xylose. Acid is not produced with *N*-acetyl-glucosamine, D-adonitol, amidon, amygdalin, D-arabinose, D-arabitol, L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D-fucose, L-fucose, gentiobiose, gluconate, D-glucose, glycerol, inositol, inulin, 2-ketogluconate, D-lyxose, D-mannitol, D-mannose, D-melezitose, D-melibiose, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, methyl-β-D-xylose, L-rhamnose, D-ribose, salicin, D-sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol, or L-xylose. The predominant cellular fatty acids of strain DG7A^T are iso-$C_{15:0}$ (24.87 %), anteiso-$C_{15:0}$ (21.42 %), and summed feature 3 ($C_{16:1}$ $\omega 7c$ and/or $C_{16:1}$ $\omega 6c$) (15.01 %). Strain DG7A^T has phosphatidylethanolamine (PE) as the major polar lipid and a G+C content of 63.5 mol%. The type strain DG7A^T (= KCTC 32554^T =

KEMB 9004-164^T = JCM 30007^T) was isolated from a soil sample collected in Seoul, (GPS; N 37° 34′ 30″ E 127° 00′ 30″), South Korea.

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