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Hymenobacter humi sp. nov., a bacterium isolated from soil

Sathiyaraj Srinivasan · Eun Sun Joo · Jae-Jin Lee · Myung Kyum Kim

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Abstract A red-pink coloured, Gram-negative, rodshaped bacterium designated as strain DG31A^T was isolated from soil collected in Seoul, South Korea. The isolate was found to grow optimally at 25 °C on R2A agar. The highest degrees of 16S rRNA gene sequence similarities of the strain were found with Hymenobacter arizonensis JCM 13504^T (98.0 %), Hymenobacter glaciei VUG-A130^T (96.1 %), Hymenobacter soli PB17^T (95.2 %), Hymenobacter antarcticus VUG-A42aa^T (94.7 %) and Hymenobacter chitinivorans $Txc1^{T}$ (92.8 %). The DNA G+C content of the novel strain, DG31A^T, was determined to be 60.8 mol%. Chemotaxonomic data revealed that the major fatty acids were summed feature 3 ($C_{16:1} \omega 7c$ and/or $C_{16:1}$ $\omega 6c$; 26.7 %), C_{16:1} $\omega 5c$ (18.9 %) and anteiso-C_{15:0} (12.9 %); the major polar lipid was identified as phosphatidylethanolamine; the polyamine pattern was found to contain sym-homospermidine; and the major quinone was identified as MK-7. The DNA-DNA relatedness of strain $DG31A^{T}$ with respect to H.

arizonensis JCM 13504^T was $19.5 \pm 2.9 \%$ (reciprocal, $19.3 \pm 0.6 \%$). Based on these data, strain DG31A^T should be classified within the genus *Hymenobacter* as a novel species for which the name *Hymenobacter humi* sp. nov. is proposed, with the type strain DG31A^T (=KCTC 32523^T = JCM 19635^T).

Keywords *Cytophagaceae* · *Hymenobacter* · Taxonomy

Introduction

The genus Hymenobacter belongs to the family Cytophagaceae (Skerman et al. 1980; Stanier 1940) and the order Cytophagales (Leadbetter 1974; Skerman et al. 1980). It was first established by Hirsch et al. (1998) and emended by Buczolits et al. (2006) to accommodate Gram-negative, red-pigmented, rodshaped aerobic bacteria that have the following properties: phosphatidylethanolamine as the main phospholipid; iso- $C_{15:0}$, anteiso- $C_{15:0}$, $C_{16:1}$ $\omega 5c$, $C_{16:1} \omega 7c/C_{16:1} \omega 6c$ and/or iso- $C_{17:1}$ I/anteiso- $C_{17:1}$ B as the major fatty acids (Baik et al. 2006; Kim et al. 2008; Klassen and Foght 2011; Reddy and Garcia-Pichel 2013); MK-7 as the predominant menaquinone; and a high G+C content (55-70 mol%). At the time of writing, the genus Hymenobacter contains 33 species (http://www.bacterio.net/hymenobacter.html). The described species Hymenobacter rigui (Baik et al.

Eun Sun Joo-co-first author.

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S. Srinivasan · E. S. Joo · J.-J. Lee · M. K. Kim (⊠) Department of Bio & Environmental Technology, College of Natural Science, Seoul Women's University, Seoul 139-774, Korea e-mail: biotech@swu.ac.kr

2006), *H. soli* (Kim et al. 2008), *H. yonginensis* (Joung et al. 2011), *H. ginsengisoli* (Hoang et al. 2013), *H. koreensis* (Kang et al. 2013), *H. saemangeumensis* (Kang et al. 2013), *H. ruber* (Jin et al. 2014), and *H. swuensis* (Lee et al. 2014) were isolated from South Korea. Recently *H. arcticus* (Chang et al. 2014), *H. kanuolensis* (Su et al. 2014), and *H. qilianensis* (Han et al. 2014) were described. In this study, strain DG31A^T was characterized by a polyphasic approach, including phylogenetic, genomic and phenotypic properties. The results obtained indicated that strain DG31A^T should be assigned as a new species in the genus *Hymenobacter*, for which the name *Hymenobacter humi* sp. nov. is proposed.

Materials and methods

Isolation, culture conditions and phenotypic characterization

For the isolation of strain DG31A^T, soil samples (1.0 g) collected in Seoul (37°33'35.87"N, 126°59'58.05"E), South Korea, were suspended in 10 ml sterile water. The resulting supernatant was serially diluted and then 100 µl of each dilution was spread on plates of R2A agar (Difco, USA) and incubated at 25 °C. The purified colonies were tentatively identified by partial 16S rRNA gene sequencing and preserved in a glycerol solution (25%, w/v) at -70 °C. Strain DG31A^T was deposited into the Korean Collection for Type Cultures (KCTC 32523^T) and the Japan Collection of Microorganisms (JCM 19635^T).

Hymenobacter arizonensis JCM 13504^{T} and Hymenobacter glaciei JCM $17,225^{T}$ were obtained from the Japan Collection of Microorganisms and cultured under the same conditions for comparative testing.

Gram reactions were determined according to the non-staining method described by Buck (1982). Cell morphology was examined by light microscopy (Nikon E600) and energy-filtering transmission electron microscopy (EF-TEM, Carl Zeiss LIBRA 120), after the cells were grown on R2A agar for 2 days at 25 °C. 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 3 % (v/v) H_2O_2 solution. Growth was assessed on different

media, including Luria-Bertani agar (LB, Difco), marine agar (MA, Difco), nutrient agar (NA, Difco), R2A agar, trypticase soy agar (TSA, Difco) and 1/10 peptone iron agar (PIA, Difco) at 25 °C. Growth at different temperatures (4, 15, 20, 25, 30, 37 and 42 °C) was assessed on R2A agar, with a 3-day incubation period. Growth at various pH (5–11 at 1 pH unit) were also assessed in R2A broth (MBcell) at 25 °C. The API 20NE, API 50CH and API ZYM microtest systems were employed, according to the recommendations of the manufacturer (bioMérieux), for studying carbon source utilization and the enzyme activities of the strains.

Pigments of strain DG31A^T were extracted using 95 % ethanol and the absorption spectrum was measured between 250 and 700 nm with a UV spectrophotometer (UV-2450, Shimazu). Flexirubin-type pigments were assayed based on colour shift after exposure to 0.1 N NaOH solution (Gosink et al. 1998; Weeks 1981).

16S rRNA gene sequencing, phylogenetic analysis, DNA–DNA hybridization, and DNA G+C content

The 16S rRNA gene of strain DG31A^T was amplified from the chromosomal DNA using the universal bacterial primer set, 9F and 1492R (Weisburg et al. 1991). Sequence analysis was performed using the 27F, 785F, 800R, and 1492R universal primers from SolGent (Daejeon, Korea). The full sequence of the 16S rRNA gene was compiled with SeqMan software (DNASTAR Inc.). For phylogenetic analysis, the nearly complete sequence of the 16S rRNA gene from strain DG31A^T (1456 bp) was compared with those of other taxa using the EzTaxon-e service (Kim et al. 2012). The 16S rRNA gene sequences of the related taxa were obtained from GenBank, then edited with the BioEdit program (Hall 1999). Multiple alignments were performed using the CLUSTAL X program (Thompson et al. 1997). Pairwise distances for the neighbour-joining algorithm (Saitou and Nei 1987) were calculated according to the Kimura two-parameter model (Kimura 1980), and the phylogenetic tree was constructed in the MEGA 5 Program (Tamura et al. 2011). Bootstrap analysis with 1000 replicates was conducted to obtain confidence levels for the branches (Felsenstein 1985). The close-neighborinterchange (CNI) on random trees method with a search factor of one and a number of initial trees (random addition) of ten was applied in maximum parsimony analysis, and maximum likelihood analysis was performed with the general-time-reversible model (gamma distributed) in the MEGA 5 Program.

DNA–DNA hybridization was performed fluorometrically, according to the method developed by Ezaki et al. (1989). For determination of G+C content, genomic DNA was extracted and purified, then enzymatically degraded into nucleosides with nuclease P1 followed by alkaline phosphatase. The resultant nucleosides were then analyzed using reverse-phase high performance liquid chromatography (HPLC), as previously described previously (Mesbah et al. 1989; Tamaoka and Komagata 1984).

Chemotaxonomic characteristics

Polar lipids were extracted according to the procedures described by Minnikin et al. (1984) and identified by two-dimensional thin-layer chromatography (TLC), followed by spraying with appropriate detection reagents (Komagata and Suzuki 1987). The total lipid profile was detected by spraying with molybdophosphoric acid solution (Sigma-Aldrich, USA) followed by heating at 150 °C; aminolipids were detected by spraying with 0.2 % (w/v) ninhydrin solution, followed by heating at 105 °C for 10 min; glycolipids were detected with 0.5 % 1-naphthol in methanol/water (1:1, v/v) and sulfuric acid/ethanol (1:1, v/v), followed by heating at 120 °C for 5-10 min; phospholipids were detected by spraying with Zinzadze reagent; and phosphatidylcholine was detected by spraying with Dragendorff's reagent (Sigma-Aldrich). The polyamines of strain DG31A^T were extracted and analysed as described by Busse and Auling (1988) and Busse et al. (1997).

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), purified by TLC and subsequently analyzed by HPLC, as described previously (Collins and Jones 1981; Shin et al. 1996). In order to perform the fatty acid methyl ester analysis, cells were grown on R2A agar for 3 days at 25 °C and then two loops of the third and fourth quadrant cells were harvested. Fatty acid methyl esters (FAME) were prepared, separated and identified with the Sherlock Microbial Identification System (MIS), produced by MIDI, Inc., Newark, DE, USA (Sasser 1990).

Results and discussion

Cells of strain DG31A^T were observed to be rodshaped (Fig. 1), Gram-negative and red-pink coloured when routinely cultured on R2A agar at 25 °C. The cells were found to be able to grow on R2A agar over a temperature range of 15–30 °C, but not at 4, 37 and 42 °C. The optimum growth temperature was found to be 25 °C and cells were capable of growth at a pH range of 6–9 but only weakly at pH 10. Growth occurs on R2A, but not on LB, MA, TSA and 1/10 PIA; weak growth was observed on NA plates. The physiological characteristics of strain DG31A^T are summarized in the species description and a comparison of differential characteristics with the type strains of closely related species is shown in Table 1.

The red-pink pigment could be extracted with a solution of 95 % ethanol and was found to have absorption maxima at 319 and 482 nm (Fig. 2). Alkalization with 0.1 volumes of 0.1 M NaOH did not lead to any shift in the peak positions, indicating that strain DG31A^T does not produce flexirubin pigment. Based on the absorption maximum at 482 nm, the pigment could be assigned to the 2'-hydroxyflexixanthin series of carotenoid pigments (Klassen and Foght 2008). Klassen and Foght (2008) previously identified hydroxyflexixanthins as the major carotenoids in all analyzed *Hymenobacter* species.

The 16S rRNA gene sequence of strain DG31A^T (GenBank/EMBL/DDBJ accession number KF601296) is a continuous stretch of 1456



Fig. 1 Cell morphology of strain DG31A^T, as determined by TEM after growth on R2A agar for 2 days at 25 °C. (*scale* $bar = 0.5 \ \mu m$)

Table 1 Differential characteristics between strain DG31A ^T and closely related species species	Characteristics	1	2	3
	Size (µm long) (by TEM)	1.6–2.3	5.0	1.0-2.0
	Size (µm wide) (by TEM)	0.6-0.8	1.5	0.5–0.8
	Production of acid from glucose	_	W	_
	Growth at			
Strains 1, DG31A ^T ; 2, <i>H. arizonensis</i> JCM 13504 ^T ; 3, <i>H. glaciei</i> JCM 17225 ^T	30 °C	+	+	_
	рН 8	+	+	W
	рН 9	+	+ (-)	W
All the data were obtained in this study. All strains were found to produce acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase (C8), β -glucosidase (esculin hydrolysis), leucine arylamidase and valine arylamidase. They did not produce arginine dihydrolase, α - fucosidase, α -galactosidase, β - glucuronidase, lipase (C14), or urease. Those that data differ from the original disparitients (<i>K</i>)-escent and	pH 10	W	+	W
	рН 11	_	+	- (+)
	0.5 % NaCl	_	+	_
	1 % NaCl	_	w	_
	1/10 peptone iron agar	_	w	_
	Enzyme activity			
	N –Acetyl- β -glucosaminidase	+	+	_
	α-Chymotrypsin	+	+	_
	β -Galactosidase (ONPG)	_	+	_
	β -Galactosidase (PNPG)	_	+	+
	α-Glucosidase	W	_	_
	β -Glucosidase	+	_	_
	α-Mannosidase	_	_	W
Foght 2011: Reddy and	Naphtol-AS-BI-phosphohydrolase	W	W	_
Garcia-Pichel 2013) are	Protease (gelatin hydrolysis)	+	+	_
given in the parentheses	Trypsin	W	+	_
+ Positive, w weak positive, - negative	DNA G+C content (mol%)	60.8	70.2	58.7



Fig. 2 Absorption spectrum of pigment extracted from strain $DG31A^{T}$. Strain $DG31A^{T}$ shows the characteristic absorption peaks of carotenoids at 318.5, and 481.5 nm. Alkalization with 0.1 volume of 0.1 M NaOH did not lead to any shift in peak positions, indicating the absence of flexirubin pigment

nucleotides. Strain DG31A^T was found to belong to the family Cytophagaceae, the order Cytophagales and the class Cytophagia. The highest degrees of sequence similarities were found with two Hymenobacter species, H. arizonensis JCM 13504^T (Reddy and Garcia-Pichel 2013) (98.0 %) and H. glaciei VUG-A130^T (Klassen and Foght 2011) (96.1 %). The closest species in a different genus, Adhaeribacter terreus DNG6^T (Zhang et al. 2009) showed a lower degree of sequence similarity (89.8 %). The phylogenetic tree (Fig. 3) shows that strain DG31A^T clearly clusters with the *Hymenobac*ter species in the family Cytophagaceae, with H. arizonensis as its closest relative. Similar relationships were observed in the neighbour-joining and maximum parsimony trees (Supplementary Figs. 1, 2).

When examining the DNA–DNA relatedness values, DG31A^T exhibited low relatedness with the most closely related type strain, *H. arizonensis* JCM 13504^T

 $(19.5 \pm 2.9 \%$, reciprocal, $19.3 \pm 0.6 \%$), values lower than 70 %, which is the threshold delineating a prokaryotic genomic species (Stackebrandt and Goebel 1994; Wayne et al. 1987). Thus, our results support the placement of strain DG31A^T as the representative of a separate and previously unrecognized genomic species in the genus *Hymenobacter*. The G+C content of genomic DNA from strain DG31A^T and *H. arizonensis* JCM 13504^T were determined to be 60.8 and 70.2 mol%, respectively. The major polar lipid found in strain $DG31A^{T}$ was identified as phosphatidylethanolamine and an aminophospholipid (APL3), in agreement with the traits listed in the emended genus description (Buczolits et al. 2006); minor amounts of unidentified aminophospholipids (APL2,4,6), unidentified amino-lipids (AL1-4) and unknown polar lipids (L1-2) were also found (Fig. 4). Strain $DG31A^{T}$ has a polar lipid



Fig. 3 Maximum likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain DG31A^T and other closely related taxa. The *bar* represents 0.02

substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at the branch points

profile similar to those of other *Hymenobacter* species (Buczolits et al. 2006), however it could be differentiated from the closely related type strain *H. arizonensis* JCM 13504^{T} by some of the polar lipids (Fig. 4). The polyamine pattern contains sym-homospermidine, which is similar to the other members of the genus *Hymenobacter* (Hoang et al. 2013).

The menaquinone MK-7 was identified as the predominant respiratory quinone of strain DG31A^T, like the most closely related strain H. arizonensis JCM 13504^T (Reddy and Garcia-Pichel. 2013). The predominant cellular fatty acids of the strain were identified as anteiso- $C_{15:0}$ (12.9 %), $C_{16.1}$ $\omega 5c$ (18.9 %) and summed feature 3 (C_{16:1} $\omega 6c$ and/ or $C_{16:1} \ \omega 7c$) (26.7 %), which are the predominant fatty acids in most Hymenobacter species. The minor fatty acids detected were iso-C_{14:0}, iso-C_{15:0}, iso-C_{15:0} 3-OH, C_{16:0}, iso-C_{16:0}, iso-C_{16:0} 3-OH, anteiso-C_{15:1} A, iso-C_{16:1} H, C_{17:1} ω 6c, and summed feature 4 (anteiso-C_{17:1} B and/or iso-C_{17:1} I). Analysis of strain DG31A^T revealed smaller amounts of iso- $C_{15:0}$ (7.0 %), whereas other closely related Hymenobacter species (*H. arizonensis* JCM 13504^T and *H. glaciei* JCM 17225^T) have larger amounts of this fatty acid. In addition, the fatty acids iso-C_{17:0} and iso-C_{15:1} G comprised less than 1 % of the total in strain DG31A^T but were present at more than 1 % in the closely related species. Finally, strain DG31A^T has larger amounts of iso- $C_{16:1}$ H (5.5 %), whereas other closely related *Hymenobacter* species have smaller amounts of this fatty acid (Table 2).

Based on the phylogenetic, phenotypic, genomic and chemotaxonomic characteristics, we conclude that strain $DG31A^{T}$ is the representative of a novel species, for which the name *Hymenobacter humi* sp. nov. is proposed.

Description of Hymenobacter humi sp. nov.

Hymenobacter humi (hu'mi L. gen. n. humi, of/from the ground/soil)

When grown on R2A agar (Difco) for 3 days at 25 °C, the cells present a rod-shaped morphology, 0.6–0.8 μ m wide and 1.6–2.3 μ m long, and are Gramnegative. Colonies are red pink-coloured. Oxidase and catalase positive. Able to grow over a temperature range of 15–30 °C but not at 37 and 42 °C. Growth is weak on NA agar but does not occur on LB, TSA, MA and 1/10 PIA at 25 °C. Not tolerant to NaCl and cannot reduce nitrate to nitrite or nitrogen. In addition, acid is not produced from D-glucose, and indole is not produced (API 20NE tests). Tests with the API ZYM system are positive for *N*-acetyl- β -glucosaminidase, acid phosphatase, alkaline phosphatase, α -chymotrypsin, cysteine arylamidase, esterase (C4), esterase (C8), α -glucosidase (starch hydrolysis), β -



Fig. 4 Two dimensional TLC sprayed with molybdophosphoric acid reagent to identify total polar lipids of strain DG31A^T (**a**) and *H. arizonensis* JCM 13504^T (**b**). Ascending solvent system: (I) chloroform/methanol/water (65:25:4, v/v/v); (II) chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). Molybdophospholic acid (PE, PG, and PL), ninhydrin (PE), α -naphthol (GL) and Zinzadze reagents (PE, PG, and PL) were

applied to detect the polar lipids. *PE* phosphatidylethanolamine, AL_X unknown aminolipids, APL_X unknown aminophospholipids, GL_X unknown glycolipids, Lx unknown polar lipids (not stainable with any of the specific spray reagents applied, indicating that it does not contain a phosphate group, an amino group, or a sugar moiety)

Table 2 Cellular fatty acid profiles of strain DG31A^T and closely related species

Fatty acids	1	2	3
Saturated			
Iso-C _{14:0}	2.1	tr	tr
Iso-C _{15:0}	7.0	12.6	14.6
Anteiso-C _{15:0}	12.9	15.1	16.4
Iso-C _{15:0} 3-OH	2.0	1.2	1.2
C _{16:0}	4.3	6.4	tr
Iso-C _{16:0}	4.6	3.3	7.2
Iso-C _{16:0} 3-OH	1.8	1.0	tr
Iso-C _{17:0}	tr	2.5	3.4
Anteiso-C _{17:0}	tr	1.0	tr
C _{17:0} 2-OH	tr	1.2	1.3
Iso-C _{17:0} 3-OH	tr	tr	1.2
Unsaturated			
Iso-C _{15:1} G	tr	1.4	9.0
Anteiso-C _{15:1} A	1.1	tr	3.2
Iso-C _{16:1} H	5.5	2.7	1.5
$C_{16:1} \omega 5c$	18.9	16.3	12.5
$C_{17:1} \omega 6c$	1.0	tr	tr
^a Summed feature 3 (C _{16:1} ω 7 <i>c</i> and/or C _{16:1} ω 6 <i>c</i>)	26.7	22.4	18.4
^a Summed feature 4 (anteiso-C _{17:1} B and/or iso-C _{17:1} I)	5.2	6.5	4.4

Strains: 1, strain DG31A^T; 2, H. arizonensis JCM 13504^T; 3, H. glaciei JCM 17225^T

All data were obtained from this study. Only fatty acids present at more than 1 % in at least one of the strains are shown. For unsaturated fatty acids, the position of the double bond was located by counting from the methyl (ω) end of the carbon chain. The *cis* isomer is indicated by the suffix *c*

tr trace amounts (<1.0 %)

^a Summed features contain fatty acids which could not be separated by GLC with the Microbial Identification System (MIDI)

glucuronidase, leucine arylamidase, naphtol-AS-BIphosphohydrolase, trypsin and valine arylamidase but are negative for, α -fucosidase, α -galactosidase, β galactosidase (OPNG), β -glucosidase, lipase (C14) and α -mannosidase. Acid is produced (in the API 50CH tests) with esculin ferric citrate and 5-ketogluconate but not with N-acetyl-glucosamine, p-adonitol, amidon, amygdalin, D-arabinose, L-arabinose, arbutin, D-arabitol, L-arabitol, D-cellobiose, dulcitol, erythritol, D-fructose, D-fucose, L-fucose, D-galactose, gentiobiose, gluconate, D-glucose, glycerol, glycogen, inositol, inulin, 2-ketogluconate, D-lactose, D-lyxose, Dmaltose, D-mannitol, D-mannose, D-melezitose, Dmelobiose, methyl- β -D-xylose, methyl- α -D-mannopyranoside, methyl-a-D-glucopyranoside, D-raffinose, Lrhamnose, D-ribose, salicin, D-saccharose, D-sorbitol, L-sorbose, D-tagatose, D-trehalose, D-turanose, xylitol, D-xylose or L-xylose. The major fatty acids are anteiso- $C_{15:0}$, $C_{16:1}$ $\omega 5c$, and summed feature 3 ($C_{16:1}$

 $\omega 6c$ and/or C_{16:1} $\omega 7c$), while the major polar lipid is phosphatidylethanolamine. MK-7 is the predominant quinone. The polyamine is sym-homospermidine. The G+C content of the type strain is 60.8 mol%.

The type strain DG31A^T (= KCTC 32523^T = JCM 19635^T) was isolated from soil in Seoul, South Korea. The NCBI GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DG31A^T is KF601296.

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