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Hymenobacter nivis sp. nov., isolated from red snow in Antarctica

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Running head: *Hymenobacter nivis* sp. nov.

Subject category: New taxa: *Bacteroidetes*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P3^T is LC153096.

Summary

A novel aerobic bacterial strain P3^T was isolated from a red snow obtained from Antarctica. Cells of strain P3^T were rod-shaped, non-motile, catalase-negative, oxidase-positive, and Gram-stain-negative. Growth was observed at temperatures ranging from 0 to 25°C, and the optimum growth was observed at 15°C. The range of pH for growth was 5.3–7.8. The G+C content of genomic DNA was 55.0 mol%. The major components in fatty acid profile were iso-$C_{15:0}$, summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B), anteiso-$C_{15:0}$, and summed feature 3 (iso-$C_{16:1}$ω7c and/or iso-$C_{16:1}$ω6c). The predominant isoprenoid quinone was MK-7. Phylogenetic analysis based on 16S rRNA gene sequence indicated that the novel isolate was a member of the genus *Hymenobacter*, and the strain showed highest sequence similarity (94%) with *H. glaciei* VUG-A130^T, *H. soli* PB17^T, and *H. antarcticus* VUG-A42aa^T. On the basis of its phylogenetic and phenotypic properties, strain P3^T (=DSM 101755^T = NBRC 111535 ^{T}) is proposed as the type strain of a new species, *Hymenobacter nivis* sp. nov.

In high-altitude or high-latitude areas, red-colored snow is occasionally observed and commonly called “red snow”. The red-coloring is caused by bloom of cold-adapted phototrophs, and various microorganisms are coexistent with them. In a previous study of red snow found in coastal Antarctica, members of the genus *Hymenobacter* belonging to the family *Cytophagaceae* were detected as predominant lineage of bacteria (Fujii *et al*., 2010). The type species and some other species of this genus were isolated from Antarctica (Hirsch *et al*. 1999; Klassen & Foght 2011), but the 16S rRNA gene sequences detected in the red snow were distinct from these species. In this study, a novel member of the genus *Hymenobacter* was isolated from the same red snow sample of Antarctica.

The strain P3^{T} was isolated from a sample of red snow obtained in 2006 (Fujii *et al*., 2010), from Yatude Valley, located in Antarctic Specially Protected Area (ASPA) No.141. The thawed snow sample was streaked on a plate of 10-fold-diluted R2A medium (Daigo) solidified with 1.5% agar, and incubated at 8°C. The strain was obtained by repeated streaking on the plates of same medium at 8°C.

Purity of the isolate was routinely checked by phase contrast light microscopy and sequencing of the 16S rRNA gene fragments. The strain was routinely cultivated with

R2A broth, and cultures grown at 15°C were used for the characterizations. The Gram-stain test was conducted with a kit (Fluka). Activities of oxidase and catalase were tested with a test reagent (bioMérieux) and 3% H_2O_2 solution, respectively. The genomic G+C content of the DNA was determined with the HPLC methods as descried previously (Katayama-Fujimura *et al*., 1984). The fatty acid analysis was performed by using the Sherlock Microbial Identification System Version 6.0, with database TSBA6 (MIDI). Analyses of polar lipids and isoprenoid quinones were carried out by the DSMZ Identification Service.

Effect of temperature on growth was tested by culturing at various temperatures (0, 2, 5, 8, 13, 15, 18, 22, 25, 28, and 30°C) in R2A broth. Effect of pH on the growth was tested with R2A supplemented with 20 mM of buffering reagent required amount of NaOH to adjust pH. The buffering reagents used were MES (pH 5.2, 5.3, 5.4, 5.5, 5.6, 5.7 and 6.3), MOPS (pH 6.7, 6.8, 6.9, 7.0, 7.2, 7.4 and 7.6), or Tricine (pH 7.8, 8.0 and 8.2). Effect of salt concentration on growth was with R2A supplemented with varying concentrations of NaCl (0, 0.5, 1, 2, 3, 4 and 5% w/v). The tests of pH and salt concentration were performed at 15°C.

Anaerobic growth was tested with anoxic R2A broth prepared by bubbling with N_2 gas, with or without addition of nitrate (10 mM).

Tests with the API 20E, API 20NE and API ZYM (bioMérieux) were performed generally according to the manufacturer's instructions. Cells were harvested by centrifugation, and incubation was performed at 15°C. The strips of API 20E and API 20NE were read after 48 hour incubation, and API ZYM strip was read after 4 hour incubation. Utilization of carbon sources was determined by using GN2 MicroPlate (Biolog) as described below. Cells collected by centrifuge were washed twice with PBS buffer, and then suspended in GN/GP-IF (Biolog). The cell suspension (transmittance levels of *ca*. 63%) was inoculated to the GN2 plates and incubated at 15°C for 5-7 days until the results were obtained.

Fragment of 16S rRNA gene was amplified with the primer pair 27F and 1492R (Lane, 1991), and the resulting PCR product was sequenced. Phylogenetic analyses were conducted using MEGA version 6 (Tamura *et al*., 2013). The obtained sequence of strain P3^T was aligned with those of relatives, and genetic distances were calculated with Kimura 2-parameter model with gamma distribution, as selected by using the model selection function in the MEGA. All positions with gaps were excluded from the calculation, and 1238 positions were included in the final dataset. Phylogenetic trees were constructed by using the methods of minimum evolution and neighbor-joining.

The cells of P3^T were non-motile Gram-negative rods (2–10 μm long and 0.6–1.0 μm

wide). Spore formation was not observed. Colonies on R2A agar plate were pink in color. The strain was catalase-negative and oxidase-positive (Table 1). The G+C content of the genomic DNA was 55.0 mol%. As respiratory quinone, only MK-7 was detected. Polar lipid profile of strain $P3^T$ analyzed by DSMZ is shown in Fig. 1.

As shown in Table 2, major components in the fatty acid profile were iso-$C_{15:0}$ (33.1%), summed feature 4 (iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B; 14.4 %), anteiso-$C_{15:0}$ (13.5%) and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; 11.9 %). The other fatty acids detected were $C_{16:1}\omega5c$ (8.0%), $C_{16:0}$ (4.8%), iso-$C_{15:0}$ 3-OH (3.2%), iso-$C_{17:0}$ (2.2%), summed feature 1 (iso-$C_{15:1}$ H and/or $C_{13:0}$ 3-OH; 1.8 %), iso-$C_{16:1}$ H (1.5%), iso-$C_{17:0}$ 3-OH (1.1%), iso-$C_{14:0}$ (1.1%), iso-$C_{16:0}$ (1.1%), iso-$C_{16:0}$ 3-OH (0.8%), $C_{15:0}$ 2-OH (0.6%), $C_{14:0}$ (0.6%), summed feature 9 (iso-$C_{17:1}$ $\omega6c$ and/or $C_{16:0}$ 10-methyl I; 0.3 %) and anteiso-$C_{17:0}$ (0.3%).

Growth of the strain was observed at temperatures 25°C or lower, and the optimum was 15°C. The range of pH for growth was 5.3–7.8 and optimum growth was observed at pH range of 6.7–7.4. No growth was observed in the medium containing 1% or higher NaCl, and 0.5% NaCl exhibited negative effect on growth of the strain. Anaerobic growth was not observed with or without nitrate.

In the test of API ZYM, activities of alkaline phosphatase, leucine arylamidase, valine

arylamidase, cystine arylamidase, acid phosphatase, α-glucosidase and *N*-acetyl-β-glucosaminidase were detected. Weak activities of esterase (C4), esterase lipase (C8), lipase (C14), trypsin, o-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-galactosidase and β-glucosidase were also observed. Activities of the other enzymes, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase were not detected. In the tests API 20NE of API 20E, reduction of nitrates to nitrite, hydrolysis of aesculin, hydrolysis of gelatin, *p*-nitrophenyl-β-D-galactopyranosidase activity, and tryptophan deaminase activity were detected. No growth or acid production was observed in tests of API 20E and API 20NE, although some of substrates in these strips were utilized in the test of GN2 plate. These discordances suggest that some of the negative results with the API strips might be attributed to unspecified factor other than substrates, and these strips are not sufficient to test growth substrates of this strain. The results of GN2 plate and other physiological characteristics are summarized in the species description and Table 1.

The 16S rRNA gene sequence analysis revealed that strain P3^{T} is a member of the genus *Hymenobacter*. The novel strain exhibited high similarity (99.8%) to a clone of 16S rRNA gene detected in the red snow sample of isolation source (Fujii *et al*., 2010). Among the type strains of *Hymenobacter* species with validly published name, *H.*

glaciei VUG-A130^T, *H. soli* PB17^T and *H. antarcticus* VUG-A42aa^T showed highest sequence similarities to strain P3^T, 94%. In the phylogenetic analysis, an identical tree topology was obtained by the methods of minimum-evolution and neighbor-joining in which strain P3^T represented an independent lineage in the genus *Hymenobacter* (Fig. 2). This tree indicated that none of existing species can accommodate this strain adequately.

In addition to the independent phylogenetic position and low sequence similarities (94% or lower) to the other species, physiological properties of the novel strain were distinct from the type strains of the related species (Table 1). On the basis of phylogenetic and phenotypic properties, strain P3^T is proposed to be assigned to a new species of the genus *Hymenobacter*, with the name *Hymenobacter nivis* sp. nov.

Description of *Hymenobacter nivis* sp. nov.

Hymenobacter nivis (ni'vis. L. gen. n. *nivis* of snow).

Cells are rod-shaped, 2–10 μm in length and 0.6–1.0 μm in width. Aerobic and non-motile. Growth occurs at temperatures between 0 and 25°C, with optimum growth at 15°C. The pH range for growth is 5.3–7.8, and optimum growth occurs at a pH range of 6.7‑7.4. The G+C content of genomic DNA is 55.0 mol%. Biolog GN2 tests show

that the type strain oxidizes *α*-cyclodextrin, glycogen, D-galactose, gentibiose, *α*-D-glucose, *α*-D-lactose, maltose, D-melibiose, *β*-Methyl-D-Glucoside, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, acetic acid, formic acid, L-alaninamide, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-ornithine, L-proline and L-threonine. Does not oxidize dextrin, Tweens 40 or 80, *N*-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, *i*-erythritol, D-fructose, L-fucose, *myo*-inositol, lactulose, D-mannitol, D-mannose, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, xylitol, succinic acid mono-methyl ester, cis-aconitic acid, citric acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, *α*-, *β*- or *γ*-hydroxybutyric acids, *p*-hydroxyphenylacetic acid, itaconic acid, *α*-ketobutyric acid, *α*-ketoglutaric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, D-alanine, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglutamic acid, D-serine, L-serine, DL-carnitine, *γ*-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-*α*-glycerol phosphate, *α*-D-glucose 1-phosphate or D-glucose-6-phosphate. In the test of API ZYM, positive for alkaline phosphatase,

esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-glucosidase (2-naphthyl-β-D-galactopyranosidase), and weakly positive for valine arylamidase. The type strain P3^T (=DSM 101755^T = NBRC 111535^T) was isolated from red snow of Antarctica.

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Table 1. Differential characteristics of strain P3^T and type strains representing related species. Strains: 1, P3^T (this study); 2, *Hymenobacter arizonensis* OR362-8^T (Reddy & Garcia-Pichel, 2013); 3, *Hymenobacter glaciei* VUG-A130^T (Klassen & Foght 2011); 4, *Hymenobacter terrenus* MIMtkLc17^T (Tang *et al.*, 2015); 5, *Hymenobacter saemangeumensis* GSR0100^T (Kang *et al*. 2013); 6, *Hymenobacter antarcticus* VUG-A42aa^T (Klassen & Foght 2011); 7, *Hymenobacter soli* PB17^T (Kim *et al*., 2008). Data of all strains were taken from their original descriptions shown above, except for enzyme activities and carbon source utilizations of strains 6 and 7 taken from Tang *et al.*, 2015. W, weak; NR, not reported.

	1	2	3	4	5	6	7
Temperature (°C)	0-25	15-30	4-28	2-35	15-30	4-18	4-30
NaCl (% w/v)	0-0.5	0-0.5	0-0.5	0-1	0-0.4	0-0.5	0-7
pH range	5.3-7.8	6-8	5-11	6-8	5.5-10.0	6-12	5.0-8.5
Catalase	-	+	+	+	+	-	+
Enzyme activities							
Lipase(C14)	W	-	-	+	-	+	+
Trypsin	W	NR	-	W	-	W	-
α-Chymotrypsin	W	NR	-	-	-	-	-
α-Galactosidase	-	NR	NR	+	-	-	-
β-Galactosidase	W	+	-	-	-	-	+
α-Glucosidase	+	NR	-	+	-	-	W
β-Glucosidase	W	NR	-	+	-	-	-
N-acetyl- β-glucosaminidase	+	NR	-	+	-	+	-
Utilization of carbon source							
Maltose	+	-	NR	-	-	W	+
Cellobiose	-	-	-	-	+	W	+
Sucrose	+	+	-	-	-	-	+

Turanose	+	NR	NR	-	+	-	+
Melibiose	+	-	NR	-	-	+	+
N-Acetyl-D-Glucosamine	-	NR	-	-	+	W	+
D-Mannose	-	+	-	-	+	+	+
D-Fructose	-	-	-	-	-	+	+
D-Galactose	+	+	-	-	-	+	+
Inosine	-	NR	-	-	-	+	+
D-Mannitol	-	+	-	-	-	W	+
myo-Inositol	-	NR	-	-	-	+	+
Glycerol	-	+	-	-	-	+	+
L-Alanine	+	-	-	-	-	+	+
L-Aspartic acid	+	-	-	-	-	+	+
L-Glutamic acid	+	-	-	-	-	+	+
L-Serine	-	-	W	-	-	+	+

226 Table 2. Major cellular fatty acids (more than 10% of total) of strain P3^T and type

227 strains representing related species.

228 Strains: 1, P3^T; 2, *Hymenobacter arizonensis* OR362-8^T; 3, *Hymenobacter glaciei*

229 VUG-A130^T; 4, *Hymenobacter terrenus* MIMtkLc17^T; 5, *Hymenobacter*

230 *saemangeumensis* GSR0100^T; 6, *Hymenobacter antarcticus* VUG-A42aa^T; 7,

231 *Hymenobacter soli* PB17^T. Data of strains 4, 6 and 7 were taken from Tang *et al.*, 2015.

232 Those of strains 2, 3 and 5 were taken from their original descriptions (Reddy &

233 Garcia-Pichel, 2013; Klassen & Foght 2011; Kang *et al*. 2013). TR, trace (<1%); ND,

234 not detected. Fatty acids less than 10% in all of the listed strains are not shown.

235

	1	2	3	4	5	6	7
iso-$C_{15:1}$ G	ND	2.6	10.5	5.5	10.4	TR	2.8
iso-15:0	33.1	21.9	13.5	20.6	28.4	20.6	25.4
anteiso-15:0	13.5	10.6	19.6	7.0	3.2	5.3	7.1
$C_{16:0}$	4.8	7.7	3.1	3.5	1.2	14.9	4.9
$C_{16:1}\omega5c$	8.0	17.6	4.6	11.2	6.5	10.1	8.8
Summed feature 3	11.9	17.0	20.3	19.2	13.2	26.4	25.2
Summed feature 4	14.4	6.7	6.6	6.8	20.2	4.9	3.3

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239 Figure legends

240

241 Fig. 1 Polar lipid profile of strain P3^T visualized by two dimensional silica gel thin layer

242 chromatography. L, lipids; PL, phospholipid; AL, aminolipids; PhAL,

243 phosphoaminolipid; PE, phosphatidylethanolamines.

244

245 Fig. 2 Minimum-evolution tree showing the phylogenetic position of P3^T within the

246 genus *Hymenobacter* based on the 16S rRNA gene sequence analysis. *Flavobacterium*

247 *aquatile* is included as an outgroup. Neighbor-joining method yielded a tree of identical

248 topology. Numbers on nodes represent percentage values of 500 bootstrap resampling

249 (values greater than 50 are shown).