Hymenobacter Translucens sp. nov., Hymenobacter Pini sp. nov. Isolated From Soil

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Research Article

Keywords: Hymenobacteraceae, Hymenobacter, Novel species, Taxonomy, Soil bacteria

Posted Date: February 24th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1365249/v1>

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Abstract

Two novel bacterial strains BT175^T and BT728^T, were isolated from soil collected in Republic of Korea. Both strains were Gram-stain-negative, rod-shaped, and had circular, convex, and pink-colored colonies. The 16S rRNA gene sequence similarity between the strains BT175^T and BT728^T was 93.6%, indicating that they represent a distinct species. 16S rRNA sequence analysis indicated that strains BT175^T and BT728^T belong to a distinct lineage within the genus *Hymenobacter* (family *Hymenobacteraceae*, order *Cytophagales*, class *Cytophagia*, phylum *Bacteroidetes*). Strain BT175^T was closely related to *Hymenobacter persicinus* 1-3-3-3^T (97.2%, 16S rRNA gene similarity), *Hymenobacter knuensis* 16F7C-2^T (96.6%), and *Hymenobacter daejeonensis* PB105^T (96.1%). Strain BT728^T was closely related to *Hymenobacter rigui* KCTC 12533^T (98.4%), *Hymenobacter metallilatus* 9PBR-2^T (97.6%), and *Hymenobacter perfusus* LMG26000^T (97.4%). Strain BT175^T and BT728^T were found to have the MK-7 as the major respiratory quinone. The DNA G + C content of strain BT175^T was 61.6 mol% and BT728^T was 59.6 mol%, respectively. Characterization based on polyphasic analysis indicated that strains BT175^T and BT728^T represent novel species of the genus *Hymenobacter* and the name *Hymenobacter translucens* sp. nov. and *Hymenobacter pini* sp. nov. are proposed. The type strain of *Hymenobacter translucens* is BT175^T (= KCTC 72330^T = NBRCxxxxx^T) and *Hymenobacter pini* is BT728^T (= KACC 22629^T = NBRCxxxxx^T).

Introduction

The genus *Hymenobacter* is a member of the family *Hymenobacteraceae* in the class *Cytophagales*. The genus *Hymenobacter* is currently confirmed to consist of 92 species with validly published names (February 2022) (<https://lpsn.dsmz.de/genus/hymenobacter>). The genus was first reported by Hirsch et al. (1999) and subsequently emended by Buczolits et al. (2006) and Han et al. (2014). Most of *Hymenobacter* species have been isolated from soil (Lee et al. 2020; Wang et al. 2020; Jang et al. 2021). Also, they are discovered extreme environments, such as antarctica (Roldan et al. 2020; Dahal et al. 2020; Sedláček et al. 2019) and uranium mine wastewater treatment system (Chung et al. 2010).

The taxonomic position of strains BT175^T and BT728^T were identified through the results of polyphasic taxonomic analysis. The results showed that strains BT175^T and BT728^T represent novel species of the genus *Hymenobacter*, and the names *Hymenoptera translucens* sp. nov. and *Hymenobacter pini* sp. nov. are respectively proposed.

Materials And Methods

Strain isolation

The strains BT175^T and BT728^T were isolated from soil samples collected at Uijeongbu city (37°44'23"N, 127°1'28"E) and Samcheok city (37°12'39.5"N, 129°5'7.1"E) in Republic of Korea, respectively. The 1 g of

soil sample was serially diluted in 9mL distilled water, and 100 µl of the diluted suspension was spread on Reasoner's 2A (R2A) agar medium (Difco). And then the plates incubated at 25°C for a week. To obtain pure colonies, each single colonies were purified by transferring colony onto new R2A agar plates under the same conditions. The novel bacterial cultures were routinely cultured on R2A agar at 25 °C and stocked in 25% (w/v) glycerol suspension at -80 °C.

Phenotypic characteristics

Cell morphologies of the strains BT175^T and BT728^T were observed using transmission electron microscopy (JEOL, JEM1010). Catalase and oxidase activity were examined by using 3% (w/v) H₂O₂ solution and 1% (w/v) tetramethyl-p-phenylenediamine diamine, respectively (Cappuccino and Sherman 2002). The Gram-staining reaction was done using a kit in accordance with the manufacturer's instructions (bioMérieux). Growth tests were assessed different temperatures (at 10-30 °C, 5 °C intervals), various pH conditions (5 to 9, 1 pH intervals) and different effect of NaCl concentrations (1%to 5% [w/v %], 1% intervals) under R2A media. Biochemical features of the strains were determined using the API 20NE, API ZYM kit in accordance with the manufacturer's instructions (bioMérieux).

Phylogenetic analysis

The genomic DNA was extracted from strains BT175^T and BT728^T using a commercial genomic DNA extraction kit (SolGent, Daejeon, South Korea). The 16S rRNA genes were amplified using the universal primers 9F and 1512R (Weisburg et al. 1991). The PCR products were purified and sequenced using universal primers 27F, 785F, 907R and 1492R (MacroGen). The 16S rRNA gene sequences similarities and taxonomic positions were determined by EzBioCloud server (<https://www.ezbiocloud.net/>) (Yoon et al. 2017). Subsequently, the 16S rRNA gene sequences of the closely related species were aligned with the strains by using Clustal X 2.0 (Larkin et al. 2007). The phylogenetic trees performed using the MEGAX program (Kumar et al. 2018) with the neighbor-joining (NJ) (Saitou and Nei 1987), maximum-likelihood (ML) (Felsenstein 1981), and maximum-parsimony (MP) algorithms (Fitch 1971). Evolutionary distances were calculated according to the Kimura two-parameter model (Kimura 1983). The stability of the tree topologies was evaluated using bootstrap analysis described in the 1,000 resampling method (Felsenstein 1985).

Genome sequencing

The DNA library was prepared with the Nextera DNA Flex Library Prep Kit (Illumina) in accordance with the manufacturer's instructions. Whole-genome sequencing (WGS) was performed using an iSeq 100 (150-bp paired-end). The genome sequencing result was assembled using the SPAdes software version 3.10.1 (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences). The genome sequences of BT175^T and BT728^T were deposited in GenBank (www.ncbi.nlm.nih.gov) database and annotated using the National Center for Biotechnology Information Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al. 2016). The average nucleotide identity (ANI) calculated by using the EzBioCloud (<https://www.ezbiocloud.net>). The digital DNA-DNA

hybridization (dDDH) values were performed using Genome-to Genome Distance Calculator (GGDC) based on formula 2 (Meier-Kolthoff et al. 2013).

Chemotaxonomic characterization

Strains BT175^T and BT728^T cultured on R2A agar were harvested and freeze-dried for the analysis of the chemotaxonomic characters. The respiratory quinone were extracted from 25 mg of dried cells using hexane, purified by thin layer chromatography, and examined by high performance lipid chromatography (HPLC) follow the previous methods (Hiraishi et al. 1996). The total lipids, glycolipids, phosphatidylcholine, and amino groups were examined using two-dimensional thin-layer chromatography (TLC). The polar lipid spots were detected by spraying the proper detection reagents as previously described (Komagata and Suzuki 1987; Minnikin et al. 1984). The cellular fatty acids methyl esters (FAME) of strains BT175^T and BT728^T were analyzed using the Sherlock Microbial Identification System V6.01 (MIS, database TSBA6, MIDI Inc) according to the protocol described by Sasser (1990).

Results And Discussion

Morphology, physiology, and biochemical analysis

Cells of strains BT175^T and BT728^T were grown at 25°C and pH 7.0 on R2A agar medium. Distinct features between the newly two isolated strains and reference strains were provided in Table 1. The negative reactions of strain BT175^T and BT728^T on API kits were given as a supplementary table (Table S1).

Phylogenetic analysis and whole-genome sequence analysis

Compared the strains BT175^T and BT728^T based on the 16S rRNA gene sequence similarities, the two strains belonged to the family *Hymenobacteraceae* and showed high sequence similarities with the genus *Hymenobacter*. The 16S rRNA gene sequence similarity between the strains BT175^T and BT728^T shared 93.6%, indicating that they represent a single species. Strain BT175^T was closely related to *H. persicinus* 1-3-3-3^T (97.2% 16S rRNA sequence similarity), *H. knuensis* 16F7C-2^T (96.6%), and *H. daejeonensis* PB105^T (96.1%). Strain BT728^T was closely related to *H. rigui* KCTC 12533^T (98.4%), *H. metallilatus* 9PBR-2^T (97.6%), and *H. perfusus* LMG26000^T (97.4%). The similarities of 16s rRNA sequence to type strains closely related to lineage BT175^T and BT728^T was less than 98.4% and less than 96.1% to other *Hymenobacter* species. Values of less than 98.7% are used as reference value for differentiation between bacterial species (Stackebrandt and Ebers 2006). According to the phylogenetic tree based on the NJ, ML and MP algorithm, strains BT175^T and BT728^T were included within the genus *Hymenobacter* (Fig 1, S1, and S2).

The draft genome of strain BT175^T was 4,957,124 bp (71.2×) and consisted of 4,232 protein-coding genes, 46 RNA genes (2 rRNA genes, 44 tRNA genes), and 31 pseudogenes. The draft genome of

strain BT728^T was 5,148,458bp (60.7×) and consisted of 4,543 protein-coding genes, 52 RNA genes (3 rRNA genes, 49 tRNA genes), and 35 pseudogenes. The genome sizes of the two strains were compared with closely related type strains, and the genome sizes of *H. persicinus* 1-3-3-3^T, *H. rigui* KCTC 12533^T and *H. metallilatus* 9PBR-2^T are 4,817,468 bp, 5,284,593 bp and 5,206,901 bp, respectively. The genome sequence of strain BT175^T and BT728^T have been deposited in GenBank under the accession numbers NZ_JAJERB010000000 and NZ_JAIZZH000000000. The DNA G+C content of strains BT175^T and BT728^T were 61.6 mol% and 59.6 mol%, respectively. This value was within the range of the G+C contents for the genus *Hymenobacter* (55-71 mol%) (Feng et al. 2019). The digital DNA-DNA hybridization values between strain BT175^T and BT728^T and other related type strains of genus *Hymenobacter* were less than 30.2% (Table S2), which are below the cutoff (70%) point (Meier-Kolthoff et al. 2013). Average nucleotide identity (ANI) values between strain BT175^T and BT728^T and other related type strains of genus *Hymenobacter* were less than 85.6%, respectively (Table S2). These values are lower than the ANI species threshold (95 - 96% ANI value) described by Richter and Rossello-Mora (2009).

Chemotaxonomic characterization

The fatty acid profiles of strain BT175^T and BT728^T were showed in Table S3. The fatty acid profiles of strains BT175^T and BT728^T were similar to those of the related type strains *H. knuensis* 16F7C-2^T and *H. persicinus* 1-3-3-3^T, respectively. The major cellular fatty acids of strains were summed feature 4 (C_{17:1} iso I / C_{17:1} anteiso B) and iso-C_{15:0}. Both of strains have lower amounts of C_{16:1} ω5c (4.5%, 4.2%) than those of other strains. The polar lipids of strain BT175^T consisted of phosphatidylethanolamine (PE), two unknown aminophospholipids (APL), six unknown phospholipid (PL) and six unknown lipids (L) (Fig. S4). The polar lipids of strain BT728^T included PE, two PL and four L (Fig. S5). Major polar lipid of both strains was also PE in other *Hymenobacter* species supporting the affiliation of the isolates to the genus.

Description of *Hymenobacter translucens* sp. nov.

Hymenobacter translucens (trans.lu'cens. L. part. adj. translucens, being translucent)

Cells are Gram-stain-negative, aerobic, rod-shaped, 0.9 μm in width and about 2.5 μm in length. Colonies are circular, smooth, convex, non-motile, and red-pink colored on Reasoner's 2A (R2A) agar plates after growth for 3 days at 25 °C. Growth is observed at various temperatures ranging from 10 to 30 °C (optimum at 25 °C). The pH range for growth is 6.0-8.0 (optimum pH 6.0) on R2A agar. Cells are positive for oxidase and catalase. In API 20NE test, positive for esculin hydrolysis, but negative for reduction of nitrates (NO₃) to nitrite (NO₂⁻), reduction of nitrates (NO₃) to nitrogen (N₂), indole production on tryptophan, glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis, β-galactosidase, D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. In API ZYM test, positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, and N-acetyl-β-glucosaminidase; weak positive for α-

chymotrypsin, but negative for control, lipase (C14), trypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase, and α -fucosidase. The major polar lipid is phosphatidylethanolamine (PE). The major respiratory quinone is MK-7. The major cellular fatty acids of strain BT175^T are summed feature 4 (C_{17:1} iso I / C_{17:1} anteiso B) (34.2%) and iso-C_{15:0} (10.2%). The genome-based G+C content is 61.6 mol%.

The type strain BT175^T (= KCTC 72330^T = NBRC xxxxx^T) was isolated from a soil sample collected in Uijeongbu City (37°44'23"N, 127°1'28"E), South Korea. The whole-genome sequence of strain BT175^T has been deposited in GenBank under the accession number NZ_JAJERB000000000 (4,957,124 bp). The GenBank accession number for the 16S rRNA gene sequence of strain BT175^T is OL468804 (1,443 bp).

Description of *Hymenobacter pini* sp. nov.

Hymenobacter pini (pi'ni. L. gen. n. pini, of a pine tree)

Cells are Gram-stain-negative, aerobic, rod-shaped, 0.9 μ m in width and about 2.1 μ m in length. Colonies are circular, convex, glistening, non-motile, and pink-colored on Reasoner's 2A (R2A) agar plates after growth for 3 days at 25 °C. Growth is observed at various temperatures ranging from 10 to 30 °C (optimum at 25 °C). The pH range for growth is 6.0-8.0 (optimum pH 6.0) on R2A agar. Cells are positive for oxidase and catalase. In API 20NE test, positive for esculin hydrolysis, gelatin hydrolysis, and D-maltose; weak positive for β -galactosidase, but negative for reduction of nitrates (NO₃) to nitrite (NO₂⁻), reduction of nitrates (NO₃) to nitrogen (N₂), indole production on tryptophan, glucose fermentation, arginine dihydrolase, urease, D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. In API ZYM test, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α -glucosidase, and *N*-acetyl- β -glucosaminidase; weak positive for lipase (C14), β -galactosidase, β -glucuronidase, and β -glucosidase, but negative for control, trypsin, α -chymotrypsin, α -galactosidase, α -mannosidase, and α -fucosidase. The major polar lipid is phosphatidylethanolamine (PE). The major respiratory quinone is MK-7. The major cellular fatty acids of strain BT728^T are iso-C_{15:0} (19.6 %) and summed feature 4 (C_{17:1} iso I / C_{17:1} anteiso B) (16.9%). The genome-based G+C content is 59.6 mol%.

The type strain BT728^T (= KACC 22629^T = NBRC xxxxx^T) was isolated from a soil sample collected in Samcheok City (37°12'39.5"N, 129°5'7.1"E), South Korea. The whole-genome sequence of strain BT728^T has been deposited in GenBank under the accession number NZ_JAIZZH000000000 (5,148,458 bp). The GenBank accession number for the 16S rRNA gene sequence of strain BT728^T is OL468806 (1,440 bp).

Declarations

Acknowledgements

This work was supported by a research grant from Seoul Women's University (2022) and by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR202102205).

In addition, we are grateful to Dr. Aharon Oren (The Hebrew University of Jerusalem, Israel) for helping with the etymology.

Author's contributions All authors equally contributed to this work.

Conflicts of interest All authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Code availability Not applicable

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors

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Table 1. Different characteristics of strains BT175^T and BT728^T and closely related species.

Characteristic	1	2	3	4	5	6	7	8	9
Colony color	red-pink	red-pink	red	red	pink	Pinkish-red	red-to-pinkish	red-pink	red
Size (µm long)	2.5	1.8–3.8	2.6–4.1	1.8–2.3	2.1	3.0	2.0–6.0	3.5–5	1.5
Size (µm wide)	0.9	0.9–1.2	0.8–1.0	1–1.1	0.9	1.0	1.0–1.5	1.0–1.5	0.6
Production of acid from glucose	soil	soil	water	grass soil	soil	fresh water	ore	mine	soil
Enzyme activity									
Alkaline phosphatase	+	+	+	+	+	+	-	+	+
Esterase (C4)	+	-	+	+	+	+	+	+	-
Esterase (C8)	+	+	+	+	+	+	+	+	w
Lipase (C14)	-	-	+	-	w	-	-	-	-
Cystine arylamidase	+	w	+	+	+	w	+	+	+
Trypsin	-	-	-	-	-	-	-	+	+
α-Chymotrypsin	w	-	-	-	-	-	-	-	+
Naphtol-AS-BI-phosphohydrolase	+	w	+	+	+	+	+	+	+
α-Galactosidase	-	-	-	-	-	w	-	-	w
β-Galactosidase (ONPG)	-	-	+	-	w	-	+	w	-
β-Glucuronidase	-	-	-	-	w	-	+	+	-
α-Glucosidase (starch hydrolysis)	-	-	w	+	+	+	+	+	+
β-Glucosidase	-	-	w	-	w	-	+	+	-
N-Acetyl-β-glucosaminidase	+	-	-	+	+	+	+	+	+
Assimilation									
D-Mannose	-	-	-	-	-	-	-	+	-

Taxa: 1, strain BT175^T; 2, strain BT728^T; 3, *H. persicinus* 1-3-3-3^T (Ten et al. 2019); 4, *H. knuensis* 16F7C-2^T (Ten et al. 2017); 5, *H. daejeonensis* PB105^T (Jin et al. 2018); 6, *H. rigui* WPCB131^T (Baik et al. 2006); 7, *H. metallilatus* 9PBR-2^T (Feng et al. 2019); 8, *H. perfusus* A1-12^T (Chung et al. 2010); 9, *H.*

swuensis DY53^T (Lee et al. 2014). Data of strains BT175^T and BT728^T was obtained in this study. Those of reference strains were obtained from previous papers. All strains were positive for alkaline phosphatase. All data were negative for nitrate reduction, leucine arylamidase, valine arylamidase, acid phosphatase, β -glucuronidase, α -fucosidase and α -mannosidase.

+, positive; -, negative; w, weak positive.

Figures

Figure 1

Neighbor-joining phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strains BT175^T and BT728^T with closely related validly published species. Bootstrap values (> 70%) based on 1,000 replications are shown at the branch nodes. Circles

indicate that the corresponding nodes were also recovered in the maximum-parsimony tree (Fig. S2). Triangles indicate that the corresponding nodes were also recovered in the maximum-likelihood trees (Fig. S3). Bar, 0.020 substitutions per nucleotide position. *Flexibacter elegans* IFO 15055^T were used as outgroup.

Supplementary Files

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- [Supplementarydata.docx](#)