Olsenella intestinalis sp. nov., isolated from cow feces

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Abstract
A Gram-stain-negative, anaerobic, non-motile, rod-shaped bacterium, designated as BGYT1T, was isolated from the feces of a cow in Andong, Republic of Korea. It was studied using a polyphasic method to determine its taxonomic position. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain BGYT1T formed a lineage within the genus Olsenella and was most closely related to O. umbonate KCTC 15140T (98.2%). The complete genome sequence of strain BGYT1T was 2,476,083 bp long with a G + C content of 66.9 mol% and contained 1835 genes and 8 contigs. The N50 value was 604,117 bp. There were 50 tRNAs, 6 rRNAs (5S, 16S, 23S), 1778 CDSs and 2 BGCs and 1 tmRNA. The values for ANI (76.8%), AAI (67.3%), and dDDH (22.2%) compared to the closest related species were all below the threshold for bacterial species delineation. In addition, genes encoding the cell wall degrading enzymes such as chitinases, β-1,3 glucanases, and proteases were also detected. The strain was able to grow at pH 6.0–8.0 (optimum, pH 7.0), in the presence of 0.5–1.5% NaCl (optimum, 0.5%, w/v) and at the temperature range of 35–40 ºC (optimum, 35 ºC). The predominant fatty acids were C16:0 DMA (20.2%), C16:0 (20.2%), C18:0 (10.5%) and C18:1 cis 9 (17.0%). The polar lipids consisted of an unidentified phospholipid, four unidentified glycolipids and three unidentified lipids. Based on its phenotypic analyses, phylogenetic and physiological characteristics, strain BGYT1T represented a novel species within the genus Olsenella, for which the name Olsenella intestinalis sp. nov. is proposed. The type strain is BGYT1T (= KCTC 25379T = GDMCC 1.3011T).

Keywords 16S rRNA sequence · Polyphasic approach · Taxonomy · Novel species

Abbreviations
TSAB  Tryptic soy agar with 5% of sheep blood

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Introduction
The bacterial genus of Olsenella, belonging to the family Atopobiacae, was first described by Dewhirst et al. (2001), with Olsenella uli as type species. Subsequently, Kraatz et al. (2011) added some phenotypic characteristics to the genus description and described a new species Olsenella umbonata. Genome-based phylogenetic classification of the phylum Actinobacteria showed the genus Olsenella was paraphyletic, but no major emendation for this genus was proposed (Nouioui et al. 2018). More recently, Zgheib et al. (2021) noticed the paraphyletic nature of Olsenella and reclassified the Olsenella species. Subsequently, Lu et al. (2021) transferred Olsenella
gallinarum to genus Thermophilibacter as Thermophilibacter gallinarum. At the end of 2021, the genus Olsenella included seven species with validly published names: O. massiliensis, O. phocaensis, O. porci, O. profusa, O. uli, O. umbonate and O. urinifantis (https://lpsn.dsmz.de/genus/olsenella). The genus Olsenella has been found in the healthy bovine rumen, human or pig feces, human gingival crevice, sheep rumen and pig jejunum, respectively (Tajima et al. 2000; Ozutsumi et al. 2005; Cho et al. 2006). The typical characteristics of the genus Olsenella include cells that are anaerobic, Gram stain positive and rod shaped, \( C_{18:1} \) cis 9 as the major fatty acid, and the G + C content of the DNA is 62–64 mol% (Dewhirst et al. 2006). The typical characteristics of the genus Olsenella have been proposed.

In the present study, strain BGYT\(^{\text{T}}\) was isolated from cow feces during a study investigating the gut microbial diversity of the animal. Polyphasic taxonomic analyses revealed that strain BGYT\(^{\text{T}}\) should be proposed as representing a novel species of the genus Olsenella for which the name Olsenella intestinalis has been proposed.

Materials and methods

Isolation, preservation, cultivation conditions

A sample of cow feces used for the isolation of bacteria was collected from the Andong of Republic of Korea. The samples were stored using an anaerobic pouch (GasPakTM EZ) and transported to the laboratory and stored at 4 °C. The isolation and cultivation of bacteria were performed in the anaerobic chamber (Coy Laboratory Products Inc.) filled with 7% CO\(_2\), 86% N\(_2\), and 7% H\(_2\). The sample suspended in phosphate-buffered saline of pH 6.0–8.0 was tenfold serially diluted until 10\(^{-5}\), subsequently, 150 μl of suspensions were spread on tryptic soy agar (TSA) supplemented with 5% sheep blood (TSAB). After incubation at 35 °C for 3 days, a large number of colonies were randomly selected, hand-picked with a sterile inoculation loop, subculture and purified, and then incubated at 35 °C for 2 days, a small single colony was selected, designated as BGYT\(^{\text{T}}\). Cultivated routinely on TSAB at 35 °C, cells were suspended in skim milk (10%, w/v sterilized) stored at −20 °C for 1 day and then transferred to −80 °C conditions for long-term preservation. To further analyze the strains closely related to BGYT\(^{\text{T}}\), two of the closely related phylogenetic relatives to strain BGYT\(^{\text{T}}\) based on 16S rRNA gene sequence comparative analysis were selected and maintained under the same conditions. These reference strains included O. umbonata KCTC 15140\(^{\text{T}}\) and O. profusa KCTC 15029\(^{\text{T}}\) obtained from the Korean Collection for Type Cultures (KCTC).

Genomic analyses

The isolated strain was identified using 16S rRNA gene sequencing. Genomic DNA was extracted from strain BGYT\(^{\text{T}}\) grown on tryptic soy agar with 5% of sheep blood (TSAB). The 16S rRNA gene of strain BGYT\(^{\text{T}}\) was amplified by 4 kinds of different universal primers: 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′), 518F (5′-CCA GCA GCC GCG GTA ATA C-3′), 805R (5′-GAC TAC CAG GGT ATC TAA TC-3′), 1492R (5′-TAC GGY TAC CTT GGT ACG ACT T-3′). The nearly complete 16S rRNA gene (1423 bp) sequence of BGYT\(^{\text{T}}\) was obtained using BioEdit program (Hall 1999) and submitted to GenBank (GenBank accession no. OM533390) and the EzBioCloud database (www.ezbiocloud.net) (Yoon et al. 2017a) for initial identification of nearly related species. The 16S rRNA sequences of closely related strains were aligned using Clustal W (Thompson et al. 1994) and the phylogenetic analysis was performed using Molecular Evolutionary Genetics Analysis (MEGA) software (version 7.0) (Kumar et al. 2016) with 1000 bootstrap iterations method (Felsenstein 1985). Phylogenetic trees based on sequences of 16S rRNA gene were reconstructed following the neighbor joining (NJ), maximum likelihood (ML) and minimum evolution (ME) algorithms (Felsenstein 1981; Saitou and Nei 1987; Rzhetsky and Nei 1992). The evolutionary distances were calculated using Kimura’s two-parameter model (Kimura 1980).

Genomic DNA was extracted using a PowerSoil® Pro DNA Isolation Kit (Cat.:47014; Qiagen, Carlsbad, CA). The quality of DNA was checked with agarose gel and the integrity and quality were also determined using Qubit (NANODPOP 2000). Sequencing was performed with Illumina Nexa sequencers. Simultaneously, Nanopore DNA was further sequenced using the MinION platform of Oxford Nanopore Technologies (ONT). Sequencing libraries were prepared using the ligation sequencing kit (SQK-LSK109; ONT) following the manufacturer’s protocol (version RPB_9059_v1_revC_08Mar2018) with SPRI bead clean up (AMPure XT beads; Beckman Coulter). Sequencing was performed as multiplex runs on a MinION with MinKnow v.1.15.1 using FLO-MIN106 R9.4 flow cells. DNA G+C content of strain BGYT\(^{\text{T}}\) was calculated from the genome data. There were three kinds of methods to calculate genomic correlations between strain BGYT\(^{\text{T}}\) and closely strains of the genus Olsenella contained ANI tool (www.ezbiocloud.net/tools/ani), the Genome-to-Genome
Distance Calculation web server (http://ggdc.dsmz.de/distcalc2.php) and AAI calculation tool (http://envo-mics.ce.gatech.edu/) (Meier-Kolthoff et al. 2013; Luo et al. 2014; Yoon et al. 2017b). The genome sequence of strain BGYT1T was uploaded to the Type Strain Genome Server (TYGS), a free biosinformatics platform for a whole-genome-based taxonomic analysis (https://tygs.dsmz.de) (Meier-Kolthoff and Göker 2019). The phylogenomic tree was reconstructed using FastME 2.1.6.1 including SPR postprocessing from the genome blast distance phylogeny (GBDP) (Lefort et al. 2015). Branch support was inferred from 100 pseudo-bootstrap replicates each. All genomes including those from the present study were annotated using the same pipeline to annotate to secure the comparison. Prokka was conducted to annotate genomes and generate gff files (Seemann 2014). Functional genes within each genome were also annotated using KEGG and deciphered to pathways using KEGG Decoder (Graham et al. 2018) and KEGG-Expander (https://github.com/bjtully/ BioData/tree/master/KEGGDecoder). Rapid Annotation of microbial genomes using Subsystems Technology (RAST) was also used to validate the annotations, particularly subsystems (Overbeek et al. 2014).

Morphology, biochemical and physiologic characteristics

For biochemical and phenotypic analysis, strains were cultivated in an anaerobic chamber for 2 days at 35 °C. The cells were desiccated using a critical point dryer (SPI-Dry Conventional Critical Point Dryer), and were coated with gold by Safematic CCU-010HV high vacuum sputter, subsequently, cell morphology was obtained using a scanning electron microscope. Gram staining was determined under a light microscope using a Gram-stain kit (Difco) according to the manufacturer’s instructions. Growth at different pH tolerance (3–9, 1 pH unit interval) was measured by inoculating in pH-adjusted TSB broth and observing the OD value using the microplate reader, the TSB liquid media was adjusted using appropriate biological buffers as a reference. To determine the optimal growth temperature, cells were examined on a TSAB plate for 5 days at various temperatures (15, 20, 25, 30, 35, 40, 45 °C, in 5 °C units) and salt tolerance was estimated by the growing cell in TSAB agar plates with NaCl concentrations ranging from 0.5 to 6%. The aerobic test was measured under aerobic conditions with an anaerobic pouch (GasPak™ EZ) at 35 °C. The catalase test was verified based on bubble formation using a catalase reagent (Bio Mérieux). The oxidase test was examined based on the production of blue color by using an oxidase reagent (Bio Mérieux). Nitrate reduction and indole and urease production were examined using API 20 NE (Bio Mérieux) test. Using API ZYM test strips to determine enzyme activities and other biochemical properties were determined using Rapid ID 32A according to the manufacturer’s instructions.

For analysis of polar lipids, compounds were carried out using freeze-dried biomass prepared from cells grown in TSB broth at 35 °C for 4 days. The polar lipids spots were separated by using TLC silica gel 60 F254 (20×20) and spraying with dyes, including 50% H2SO4, 0.1% ninhydrin (Sigma-Aldrich), molybdenum (Sigma-Aldrich), which used to identify total lipids, amino lipids, and phospholipid, respectively. Cellular fatty acid profiles were determined in cells grown on TSAB agar plate under anaerobic conditions. Strain BGYT1T is deposited in the Korean Type Culture Collection (KCTC 25379T) and the Chinese Microbial Culture Collection (GDMCC 1.3011T). The fatty acids were saponified, methylated, and extracted by using the MIDI/Hewlett Packard Microbial Identification System (Piñeiro et al. 2008) based on the manufacturer’s processing manuals (Sasser 1990) and subsequently identified using gas chromatography (GC-210; Shimadzu) and Sherlock™ Chromatographic Analysis System software package with the Anaerobic database version 6.1.

Results and discussion

Phylogenetic characteristics

Comparative analysis of 16S rRNA (1423 bp) gene sequences showed that strain BGYT1T has the highest similarity with O. umbonata KCTC 15140T (98.24%), O. profusa KCTC 15029T (97.46%), O. uli DSM 7084T (96.55%), Fannyhessea vaginae DSM 15829T (94.86%), Lancefieldella parvula DSM 20469T (94.07%) and Pararactidigestivibacter faecalis KCTC 15699T (93.09%) in EzBioCloud libraries. These values were lower than the value of 98.65% for proposing novel species by Kim et al. (2014), and suggesting that strain BGYT1T represents a novel species. The phylogenetic trees reconstructed using ML, ME and NJ methods indicated that strain BGYT1T related to the species in the genus Olsenella and had different locations compared with other Olsenella species (Figs. 1, S1, S2).

Phylogenomics and genomic analyses

The phylogenetic tree based on the TYGS revealed the relationship between strain BGYT1T and the related type strains (Fig. 2), which showed that strain BGYT1T was placed in a different species branch from other Olsenella species. Comparison of genomic distances and calculation of the dDDH values between strain BGYT1T and its closest strains resulted in values below 70%, the cut-off was determined as a threshold for novel species (Meier-Kolthoff et al. 2013). The ANI values between strain BGYT1T and its closest
Fig. 1 Minimum evolution tree based on 16S rRNA gene sequences showing the relationships between strain BGYT1^T and its nearest phylogenetic neighbors. Bootstrap values from neighbor joining, maximum likelihood and minimum evolution analyses are shown (NJ/ML/ME). Eggerthella guodevinii HF-1101^T (MN203625) was used as an outgroup. Bootstrap values of > 50% based on 1000 replications are shown at branch nodes. Scale Bar = 0.01 substitution per nucleotide position.

Fig. 2 Tree inferred with FastME 2.1.6.1 from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 74.2%. The tree was rooted at the midpoint.
relatives reached 76.8%, and calculated ANI values were below the 95–96% threshold for species description, which confirmed the novelty of the species (Ciufo et al. 2018; Jain et al. 2018), respectively. AAI values and dDDH values between strain BGYT1T and *O. umbonata* revealed 67.3% and 22.2%, respectively, the obtained values are less than the 95% of AAI and 70% of dDDH threshold for bacterial species classification. (Table 1) (Konstantinidis and Tiedje 2007; Meier-Kolthoff et al. 2013; Luo et al. 2014).

The genome of strain BGYT1T contained 1835 genes and 8 contigs with a total length of 2,453,694 bp. The N50 value was 604,117 bp. There were fifty tRNAs, six rRNAs (5S, 16S, 23S), and one tmRNA. Based on the whole-genome sequence, the DNA G + C content was 66.9%. In addition, there were one thousand seven hundred and seven–eight CDSs and two BGCs (Table S1). Furthermore, function annotation of the genomes indicated that 62 genes were classified as function unknown of orthologous genes clusters, but a lot of functions known was annotated such as cell wall/membrane/envelope biogenesis, translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism, transcription, signal transduction mechanisms, nucleotide transport and metabolism, defense mechanisms, inorganic ion transport and metabolism, lipid transport and metabolism (Fig. 3). Genes encoding the cell wall degrading enzymes such as chitinases, β-1,3 glucanases, and proteases were also detected in the strain BGYT1T. Bacteria producing chitinase, glucanase and protease enzymes can be applied to control plant fungal pathogens since chitin, α- and β-glucans and glycoproteins are the major components of the cell walls of fungi (Dimkić et al. 2022).

### Table 1  
AAI, ANI and dDDH genomic comparisons between strain BGYT1T and its closest phylogenetic neighbors

| GenBank Accession number | Strain genome              | AAI (%) | ANI (%) | dDDH (%) |
|--------------------------|----------------------------|---------|---------|----------|
| GCA_900105025.1          | *O. umbonata* KCTC 15140<sup>T</sup> | 67.26   | 76.8    | 22.2     |
| GCA_000143845.1          | *O. uli* ATCC 49627<sup>T</sup>    | 66.97   | 77.04   | 21.8     |
| GCA_001457795.1          | *O. massiliensis* SIT9<sup>T</sup> | 64.11   | 75.34   | 20.7     |
| GCA_014982725.1          | *Thermophilobacter gallinarum* Cla-CZ-62<sup>T</sup> | 61.26   | 76.03   | 21.2     |
| GCA_900155635.1          | *O. urininfantis* Marseille-P3197<sup>T</sup> | 65.29   | 75.85   | 20.9     |
| GCA_900120385.1          | *O. phocaenensis* Marseille-P2936<sup>T</sup> | 64.20   | 76.99   | 23       |

![Fig. 3](image-url)  
Clusters of Orthologous Group (COG) functional classification of proteins in the genome of strain BGYT1T
Morphological, physiological and biochemical features

Cells grown on the TSAB media were circular, convex, smooth and entire after at 35 °C for 2 days. The strain BGYT1T was anaerobic, non-motile, oxidase and catalase negative. Cells were rod-shaped without flagella, cell size in range 1.5–3.1 μm x 0.2–0.3 μm (Fig. S3), growth at pH 6.0–8.0, at temperature range of 35–40 °C and salt tolerance ranges 0.5–1.5% (w/v; optimum, 0.5%). Urease was detected by API 20A test, strain BGYT1T could be obviously distinguished from the closest related strains, O. umbonsts KCTC 15140T showed higher acid production (from API 20A) and enzyme activities from API ZYM and API Rapid 32A (Table 2). Based on enzymatic activity test, the strain was positive for alkaline phosphatase, arginine and β-glucosidase and arginine dihydeolase. All other tests were negative (Table S2). The dominant fatty acids (> 10% of the total fatty acids) of strain BGYT1T were C16:0 (20.2%), C16:0 DMA (20.2%), C18:0 (10.5%), C18:1 cis 9 (17.0%), which were the same fatty acids profiles found in some type strains; e.g., O. umbonata KCTC 15140T had C16:0 (14.2%), C16:0 DMA (22.5%) and C18:1 cis 9 (19.6%), O. profusa KCTC 15029T had C16:0 (12.7%), C16:0 DMA (22.1%) and C18:1 cis 9 (6.4%). Furthermore, strain BGYT1T could be distinguished from its nearest phylogenetic neighbor O. umbonata KCTC 15140T, by a higher amount of C16:0 and an extra component of C18:2 cis 9, 12 (Table S3). In BGYT1T, phospholipid, glycolipids and lipids were detected as predominant polar lipids. An unidentified phospholipid, four unidentified glycolipids and three unidentified lipids were detected (Fig. S4). The polar lipid profiles of strain BGYT1T were similar to those of the reference strains O. umbonata KCTC 15140T and O. profusa KCTC 15029T.

Taxonomic conclusion

The values for ANI (76.8%), AAI (67.3%), and dDDH (22.2%) compared to the nearest related species were all below the threshold for bacterial species delineation. Some physiological characteristics indicated that strain BGYT1T was different from these closest strains from the API test. Due to the above information, strain BGYT1T represents a novel species of the genus Olsenella, which was given the name as Olsenella intestinalis.

Description of Olsenella intestinalis sp. nov.

Olsenella intestinalis (in.testi.na’lis. N.L. masc./fem. adj. intestinalis, pertaining to the intestine).

Colonies are circular, convex, smooth and entire, 0.2–0.3 mm in diameter on TSAB after incubation at 35 °C for 2 days. Cells are Gram-stain-positive rods with rounded ends, anaerobic, non-motile, cell size in the range 1.5–3.1 μm x 0.2–0.3 μm, oxidase and catalase negative. Growth in pH range 6.0–8.0 and temperature range 35–40 °C with optimum growth at 35 °C and pH 7.0. Cells grow well in presence of 1.5% NaCl, and 2% NaCl inhibits the growth of cells. Positive for alkaline phosphatase, β-glucosidase and arginine dihydeolase. The polar lipids consisted of an unidentified phospholipid, four unidentified glycolipids and three unidentified lipids. The major fatty acids profiles (> 10%) are contained C16:0, C16:0 DMA, C18:0 and C18:1 cis 9. The genomic DNA G+C content is 66.9%.

The type strain BGYT1T (= KCTC 25379T = GDMCC 1.3011T), was isolated from cow feces collected from the Republic of Korea (Andong).
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Author contributions Strain BGYT1T was isolated by YG and M-KL. Material preparation, data collection and analyses were performed by YG, ZL, M-JK, J-YL, HC, S-HP, SWK, J.L, JHL, HBK, EK and JHL. The manuscript was written by YG, ZL and M-KL. All authors read and approved the final manuscript.

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Data availability The GenBank accession numbers of the 16S rRNA gene and the whole-genome sequences of the strain BGYT1T are OM553390 and JALGRK0000000000, respectively.

Declarations

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

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