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 BGYT1^T, was isolated from the feces of a cow in Andong, Republic of Korea. It was studied by using a polyphasic method to determine its taxonomic position. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain BGYT1^T formed a lineage within the genus *Olsenella* and was most closely related to *O. umbonate* KCTC 15140^T (98.24%). The complete genome sequence of strain BGYT1^T was 2,476,083 bp long with a G+C content of 66.9 mol%. The values for ANI (76.8%), AAI (67.26%), and dDDH (22.2%) compared to the closest related species were all below the threshold for bacterial species delineation. 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°C (optimum, 35°C). The predominant fatty acids were $C_{16:0}$ DMA (20.15%), $C_{16:0}$ (20.15%), $C_{18:0}$ (10.54%) and $C_{18:1}$ cis 9 (16.99%). The polar lipid profiles contained phospholipid, glycolipid and five unidentified glycolipids. Based on its phenotypic analyses, phylogenetic and physiological characteristics, strain BGYT1^T represented a novel species within the genus *Olsenella*, for which the name *Olsenella intestinalis* sp. nov. is proposed. The type strain is BGYT1^T (=KCTC 25379^T=GDMCC 1.3011^T).

Introduction

The bacterial genus of *Olsenella*, belonging to the family *Atopobiaceae*, was first described by Dewhirst *et al*. (Dewhirst et al. 2001) with *Olsenella uli* as type species. Subsequently, Kraatz *et al*. (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. (Zgheib et al. 2021) noticed the paraphyletic nature of *Olsenella* and reclassified the *Olsenella* species. At the end of 2021, the genus *Olsenella* included seven species with validly published names: *O. massiliensis*, *O. phocaeensis*, *O. porci*, *O. profusa*, *O. uli*, *O. umbonate* and *O. urininfantis* (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. 2001; Göker et al. 2010; Li et al. 2015).

In the present study, strain BGYT1^T was isolated from cow feces during a study investigating the gut microbial diversity of the animal. Polyphasic taxonomic analyses revealed that strain BGYT1^T should be proposed as representing a novel species of the genus *Olsenella* for which the name *Olsenella internalis* has been proposed.

Method And Materials

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 ten-fold serially diluted until 10^{-5}, subsequently, 150µl 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 BGYT1^T. The isolated strain was identified using 16S rRNA gene sequencing. Strain BGYT1^T is deposited in the Korean Type Culture Collection (KCTC 25379^T) and the Chinese Microbial Culture Collection (GDMCC 1.3011^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 BGYT1^T, two of the closely related phylogenetic relatives to strain BGYT1^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^T and *O. profusa* KCTC 15029^T obtained from the Korean Collection for Type Cultures (KCTC).

Phylogenetic Analyses

Genomic DNA was extracted from strain BGYT1^T grown on tryptic soy agar with 5% of sheep blood (TSAB). The 16S rRNA gene of strain BGYT1^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 GTT ACG ACT T-3´). The nearly complete 16S rRNA gene (1,423 bp) sequence of BGYT1^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 (version7.0) (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 Analyses

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 was performed for this sample. Briefly, genomic 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 v1.15.1 using FLO-MIN106 R9.4 flow cells. DNA G + C content of strain BGYT1^T was calculated from the genome data. There were three kinds of methods to calculate genomic correlations between strain BGYT1^T and closely strains of 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://enve-omics.ce.gatech.edu/) (Meier-Kolthoff et al. 2013; Luo et al. 2014; Yoon et al. 2017b). The genome sequence of strain BGYT1^T was uploaded to the Type Strain Genome Server (TYGS), a free bioinformatics 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℃. The cells were desiccated by 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 ℃, in 5 ℃ 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 (GasPakTM EZ) at 35℃. 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℃ for 4 days. The polar lipids spots were separated by using TLC silica gel 60 F_{254} (20x20) and spraying with dyes, including 50% H_2SO_4, 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. The fatty acids were saponified, methylated, and extracted by using the MIDI/Hewlett Packard Microbial Identification System (Piñeiro-Vidal 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 (1,423 bp) gene sequences showed that strain BGYT1^T has the highest similarity with *O. umbonata* KCTC 15140^T (98.24%), *O. profusa* KCTC 15029^T (97.46%), *O. uli* DSM 7084^T (96.55%), *Fannyhessea vaginae* DSM 15829^T (94.86%), *Lancefieldella parvula* DSM 20469^T (94.07%) and *Paratractidigestivibacter faecalis* KCTC 15699^T (93.09%) in EzBioCloud libraries. These values were lower than the value of 98.65% for claiming novel species by Kim *et al*. (Kim et al. 2014) and suggesting that strain BGYT1^T represents a novel species. The phylogenetic trees reconstructed using ML, ME and NJ methods indicated that strain BGYT1^T related to the species in the genus *Olsenella* and had different locations compared with other *Olsenella* species (Fig. 1).

Phylogenomics And Genomic Analyses

The phylogenetic tree based on the TYGS revealed the relationship between strain BGYT1^T and the related type strains (Fig. 2), which showed that strain BGYT1^T was placed in a different species branch from other *Olsenella* species. Comparison of genomic distances and calculation of the dDDH values between the draft genomes of strains BGYT1^T 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 BGYT1^T and its closest relatives reached 76.8%, and calculated ANI values were below the 95–96% threshold for species description, to further offered the novelty of the proposed species(Ciufo et al. 2018; Jain et al. 2018), respectively. AAI values and dDDH values between strain BGYT1^T and *O.*

umbonata reached 67.26% and 22.2%, respectively. which were below the 95% AAI and 70% dDDH threshold for novel species (Table. 1) (Konstantinidis and Tiedje 2007; Meier-Kolthoff et al. 2013; Luo et al. 2014).

The genome of strain BGYT1^T contained 1,835 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 BGYT1^T. 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).

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 BGYT1^T was anaerobic, non-motile, oxidase and catalase negative. Cells were rod-shaped without flagella, cell size in range 1.5–3.1μm × 0.28–0.32μm (Fig. 4), growth at pH 6.0 to 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 BGYT1^T could be obviously distinguished from the closest related strains, *O. umbonsts* KCTC 15140^T had made more acid production (from API 20A) and enzyme activities from API ZYM and API Rapid 32A (Table. 2).

The dominant fatty acids (> 10% of the total fatty acids) of strain BGYT1^T were $C_{16:0}$ (20.15%), $C_{16:0}$ DMA (20.15%), $C_{18:0}$ (10.54%), $C_{18:1}$ cis 9 (16.99%), which was the same fatty acids profiles found in some type strains such as *O.umbonata* KCTC 15140^T had $C_{16:0}$ (14.21%), $C_{16:0}$ DMA (22.49%) and $C_{18:1}$ cis 9 (19.58%), *O. profusa* KCTC 15029^T had $C_{16:0}$ (12.67%), $C_{16:0}$ DMA (22.12%) and $C_{18:1}$ cis 9 (6.41%). Furthermore, strain BGYT1^T could be distinguished from its nearest phylogenetic neighbor *O.umbonata* KCTC 15140^T, by a higher of $C_{16:0}$ and an extra component of $C_{18:2}$ cis 9, 12 (Table. 3). The major polar lipid profiles of strain BGYT1^T were phospholipid and glycolipid (Fig. S1), and similar to those of the reference strains *O. umbonata* KCTC 15140^T and *O. profusa* KCTC 15029^T supporting the affiliation of the isolate to the genus. In addition, strain BGYT1^T also contained five unidentified glycolipids that were also detected (Fig. S1).

Taxonomic Conclusion

16S RNA sequence similarity values were below the threshold of 98.65% as well as AAI values of strain BGYT1^T were below the threshold of 95% for novel species. Furthermore, ANI and dDDH values between strain BGYT1^T and closely related strains were below the 95% ANI and 70% dDDH threshold values for delineation of species. Some physiological characteristics indicated that strain BGYT1^T was different from these closest strains from the API test. Due to the above information, strain BGYT1^T represents a novel species of genus *Olsenella*, which was given the name as *Olsenella intestinalis*.

Description of Olsenella intestinalis sp. nov.

Olsenella intestinalis (in.tes.ti.na'lis. N.L. masc./fem. adj. *intestinalis*, pertaining to the intestines, from where the type strain was isolated).

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 0.28–0.32 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 and β-glucosidase. Negative for esterase (C4), esterase lipase (C8), lipase, valine arylamidase, cysteine arylamidase, β-glucuronidase, α-glucosidase. Negative for reduction of nitrates and nitrites. Negative for production of urease and indole. Does not produce brown diffusible pigment by API 20A. Negative for utilization of glucose, mannitol, lactose, maltose, salicin, esculin ferric citrate, glycerol, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose, trehalose. Positive for arginine and β-glucosidase. Negative for serine arylamidase, arylamidase, glutamyl glutamic acid, histidine arylamidase, glycine arylamidase, alanine arylamidase, tyrosine arylamidase, pyroglutamic acid arylamidase, leucine arylamidase, phenylalanine arylamidase, leucyl glycine arylamidase, proline arylamidase, arginine arylamidase, N-acetyl- β-glucosaminidase, β-glucuronidase, α-arabinosidase, α-galactosidase, β-galactosidase, β-galactosidase 6 Phosphate. The polar lipids profiles of strain BGYT1^T comprise phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine and unidentified polar lipids. The major fatty acids profiles (> 10%) are contained $C_{16:0}$ (20.15%), $C_{16:0}$ DMA (20.15%), $C_{18:0}$ (10.54%) and $C_{18:1}$ cis 9 (16.99%). The AAI, ANI and dDDH values between BGYT1^T and its closest strain is 67.26%, 76.8% and 22.2%, respectively. The genomic DNA G + C content is 66.9 mol%.

The type strain BGYT1^T (= KCTC 25379^T = GDMCC 1.3011^T), was isolated from cow feces collected from the Republic of Korea (Andong).

Abbreviations

TSA, tryptic soy agar; TSAB, tryptic soy agar with 5% of sheep blood; TSB, tryptic soy Broth; ANI, average nucleotide identity; dDDH, digital DNA-DNA hybridization; AAI, average amino acid identity; GGDC,

genome-to-genome distance calculation.

Declarations

Data availability

The GenBank accession numbers of the 16S rRNA gene and the whole-genome sequences of the strain BGYT1^T are OM533390 and JALGRK000000000, respectively.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Tables

Table. 1 AAI, ANI and dDDH genomic comparisons between strain BGYT1^T and its closest phylogenetic neighbors.

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

Table. 2 Comparison of the biochemical differences between BGYT1^T and its closest strains in the genus of *Olsenella*. 1, *Olsenella intestinalis* BGYT1^T; 2, *Olsenella umbonata* KCTC 15140^T; 3, *Olsenella profusa* KCTC 15029^T.

Characteristics	1	2	3
ZYM			
Alkaline phosphatase	+	+	-
Esterase(C4)	-	+	-
Esterase Lipase(C8)	-	+	-
Lipase	-	+	-
Valine arylamidase	-	+	+
Cysteine arylamidase	-	+	+
β-glucuronidase	-	-	+
α-glucosidase	-	+	+
β-glucosidase	+	-	+
20A			
D-glucose	-	+	+
D-mannitol	-	-	+
D-lactose	-	-	+
Sucrose	-	+	+
Maltose	-	+	+
Salicin	-	-	+
D-xylose	-	-	+
L-arabinose	-	-	+
Gelatin	-	-	+
Glycerol	-	-	+
Mannose	-	+	-
D-melezitose	-	-	+
D-raffinose	-	-	+
D-sorbitol	-	-	+
L-rhamnose	-	-	+
D-trehalose	-	+	+
Rapid ID32 A			

Arginine dihydeolase	+	-	+
Galactosidase	-	+	-
Leucine arylamidase	-	+	+

Table. 3 Cellular fatty acid profiles of strain BGYT1^{T} and the closely related strains of the genus *Olsenella.* Major profiles (>10%) are shown in bold. 1, *Olsenella intestinalis* BGYT1^{T}; 2, *Olsenella umbonata* KCTC 15140^{T}; 3, *Olsenella profusa* KCTC 15029^{T}.

Fatty acid	1	2	3
Saturated			
$C_{10:0}$	2.81	-	-
$C_{12:0}$	-	1.45	1.57
$C_{14:0}$	5.40	5.91	**17.21**
$C_{14:0}$ DMA	-	-	3.68
$C_{16:0}$ ALDE	5.52	5.75	5.37
$C_{16:0}$ DMA	**20.15**	**22.49**	**22.12**
$C_{18:0}$ ALDE	-	1.54	-
$C_{18:0}$ DMA	4.18	3.61	3.59
$C_{16:0}$	**20.15**	**14.21**	**12.67**
$C_{18:0}$	**10.54**	7.78	6.50
Branched			
Anteiso-$C_{13:0}$	-	-	2.21
Iso-$C_{14:0}$	-	-	2.62
Anteiso-$C_{15:0}$	-	-	2.83
Unsaturated			
$C_{16:1\ CIS}$ 9	-	1.56	-
$C_{18:1\ CIS}$ 9	**16.99**	**19.58**	6.41
$C_{18:1\ CIS}$ 9 DMA	7.79	9.96	9.25
$C_{18:2\ CIS}$ 9,12	6.47	-	-
Summed Feature 1*	-	-	1.77
Summed Feature 7**	-	2.98	2.19

*Summed Feature 1 comprised $C_{14:0}$ ALDE or $C_{13:0\ cis}$ 12 FAME.

**Summed Feature 7 comprised $C_{17:0\ cis}$ 8 FAME or $C_{17:2}$ FAME.

Figures

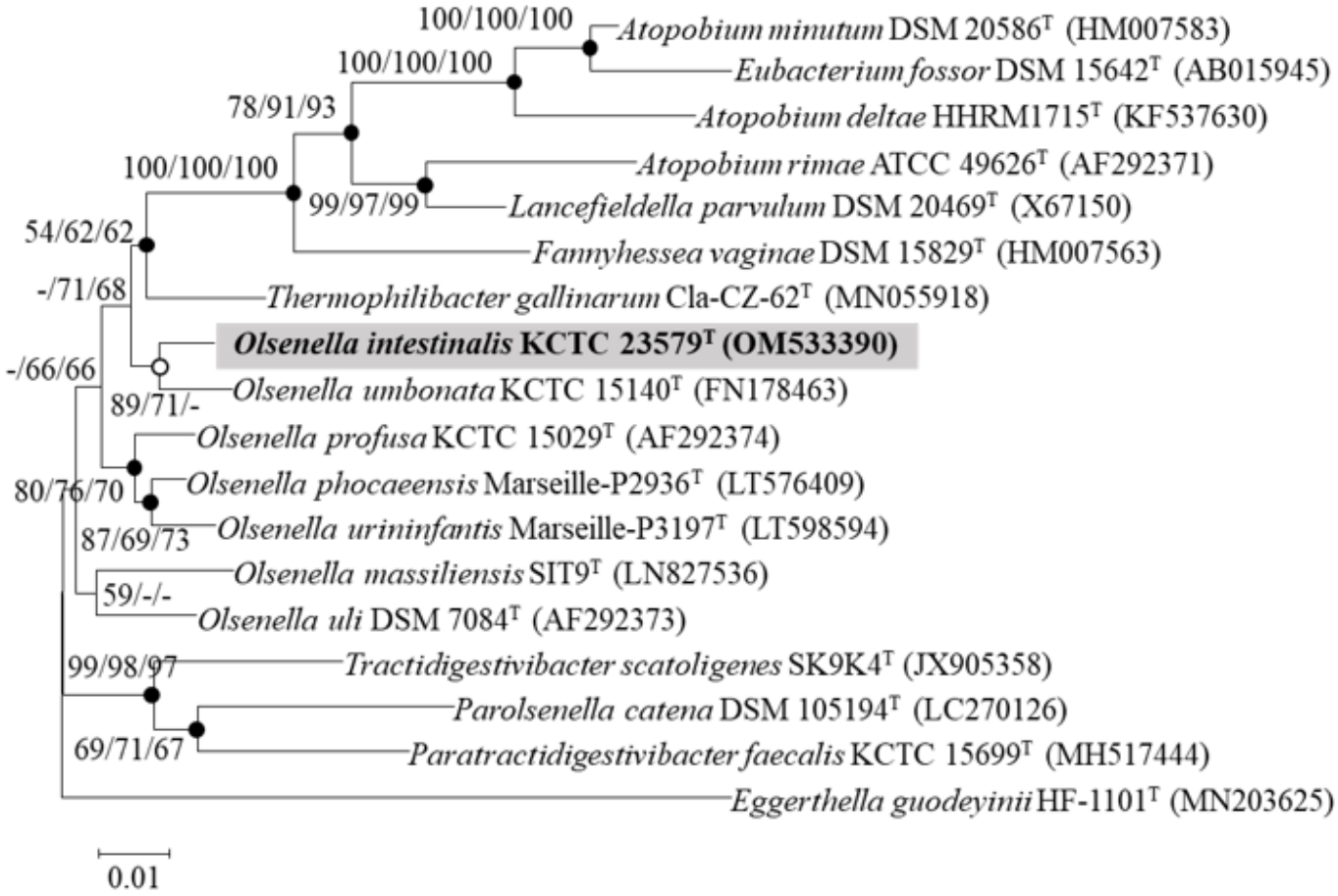

Figure 1

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 guodeyinii* 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

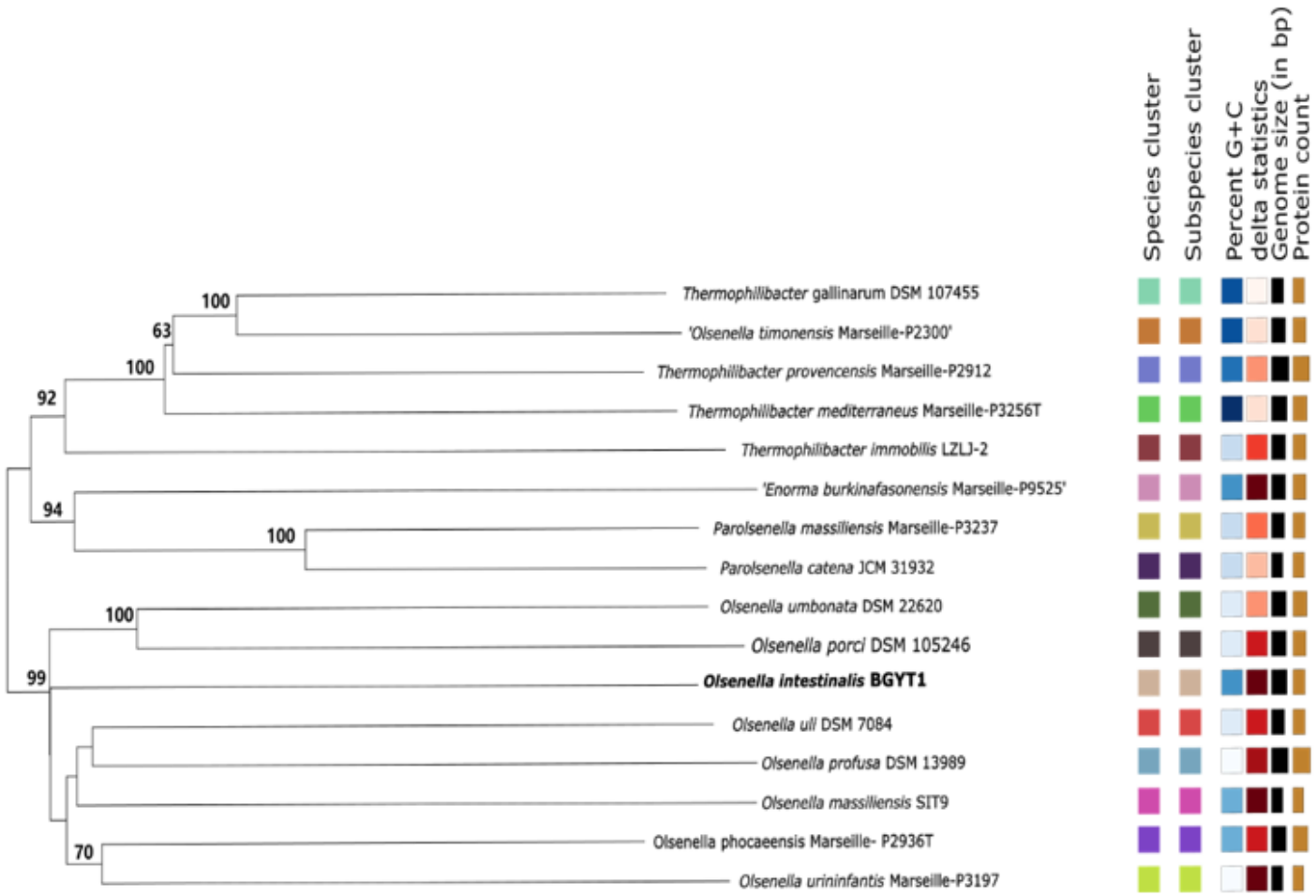

Figure 2

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.

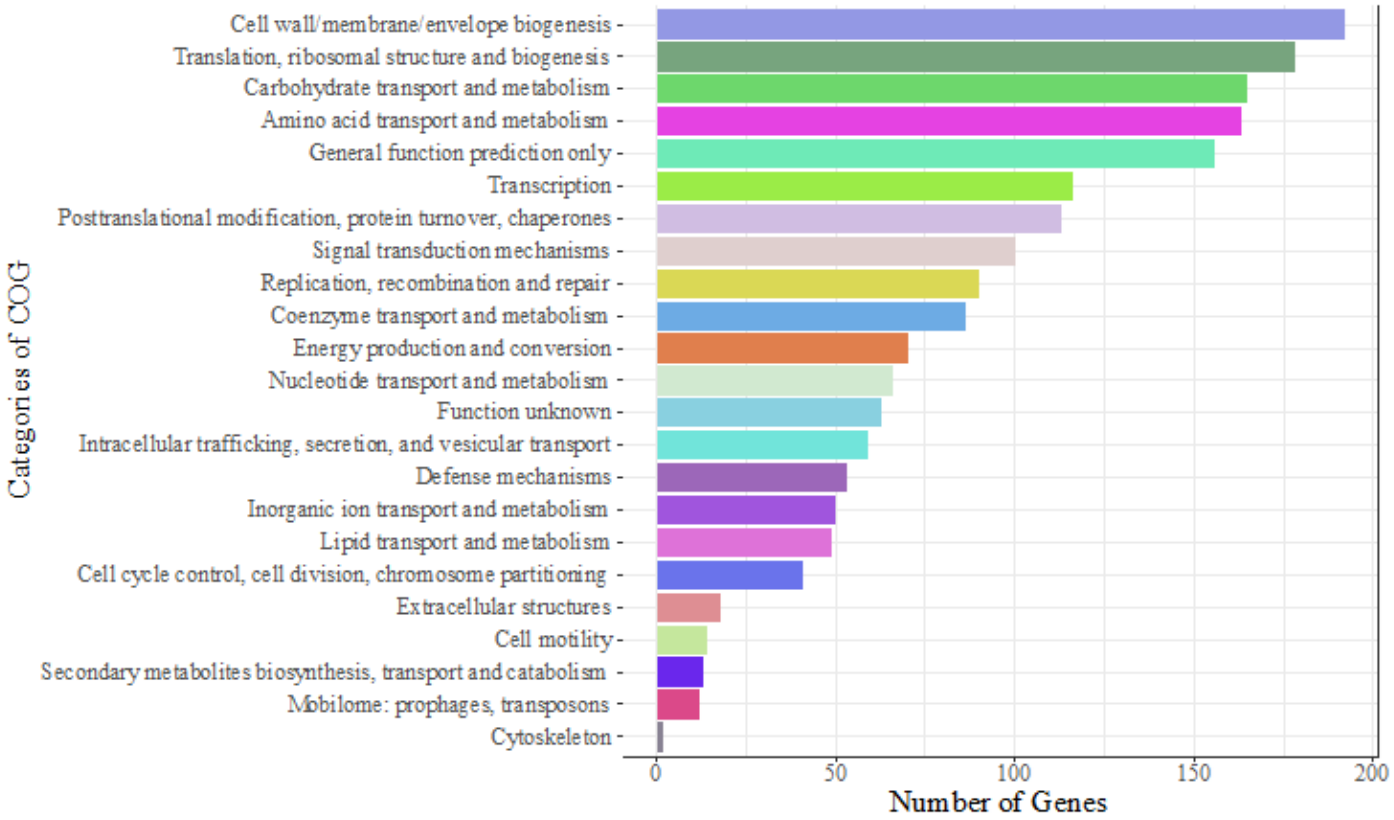

Figure 3

Clusters of Orthologous Group (COG) functional classification of proteins in the genome of strain BGYT1^{T}.

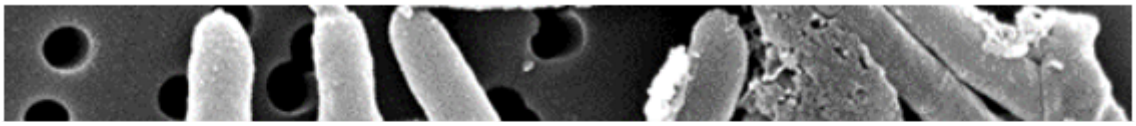

Figure 4

Scanning electron microscope (SEM) image exhibiting cell morphology of the strain BGYT1^{T}. Scale bar = 1µm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementmaterialsofBGYT11.pdf