Bifidobacterium mongoliense sp. nov., from airag, a traditional fermented mare's milk product from Mongolia

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Two novel micro-organisms, designated strains YIT 10443^T and YIT 10738, were isolated from airag, a traditional fermented mare's milk from Mongolia. The two strains were Gram-positive-staining, non-motile, asporogenous, catalase-negative, facultatively anaerobic rods of various shapes. Comparative analyses of 16S rRNA and ClpC ATPase (*clpC*) gene sequences and the presence of fructose-6-phosphate phosphoketolase (F6PPK) demonstrated that the novel strains were members of the genus *Bifidobacterium*. On the basis of 16S rRNA gene sequence similarity, the type strains of *Bifidobacterium minimum* (96.6 %) and *Bifidobacterium psychraerophilum* (95.7 %) were the closest neighbours of the novel strains, and DNA–DNA reassociation values with these strains were found to be lower than 15 %. The phenotypic and genotypic features demonstrated that the two strains represent a single, novel *Bifidobacterium* species, for which the name *Bifidobacterium mongoliense* sp. nov. is proposed. The type strain is YIT 10443^T (=JCM 15461^T =DSM 21395^T).

Bifidobacteria are Gram-positive, anaerobic, non-motile, non-spore-forming bacteria that are typically found in the intestinal tract of humans and animals (Moore & Holdeman, 1974; Scardovi & Zani, 1974; Finegold *et al.*, 1983; Scardovi, 1986; Matsuki *et al.*, 2004; Simpson *et al.*, 2004). Currently, 29 species and 9 subspecies have been described. It has been suggested that *Bifidobacterium* species are important for maintaining general health, and many attempts have been made to increase the populations of bifidobacteria in the intestinal tract of humans and animals by supplying certain bifidobacterial strains as probiotics (Fuller, 1989; Kitajima *et al.*, 1997; Lee *et al.*, 1999). Bifidobacteria have been isolated not only from the gastrointestinal tracts of humans and animals but also from

environmental materials such as sewage (Scardovi & Trovatelli, 1974) and an anaerobic digester (Dong *et al.*, 2000).

During a study on the diversity of lactic acid bacteria (LAB) in airag (fermented mare's milk) and tarag (fermented milk of cows, yaks, goats or camels), traditional fermented milk products from Mongolia, bifidobacterial strains YIT 10443^T and YIT 10738 were isolated from two of 22 airag samples, which were collected in the Mongolian provinces of Umnugobi and Uburhangai, respectively (Watanabe *et al.*, 2008). Samples were serially diluted with sterile saline (0.85 % NaCl) before being inoculated onto modified MRS agar (glucose replaced with 1 % lactose) supplemented with 0.001 % sodium azide and 0.001 % cycloheximide and incubated anaerobically at 30 °C for 3 days in an anaerobic chamber (Coy Laboratory Products; atmosphere comprising 88:5:7 N₂/CO₂/H₂). Pinpoint colonies were found initially among colonies of LAB on the surface of the agar plates. Cell counts of bifidobacterial strains YIT 10443^T and YIT 10738 in airag samples were 7.74 log₁₀ c.f.u. ml⁻¹ and 7.60 log₁₀ c.f.u. ml⁻¹, respectively. The strains were suspended in nutrient broth (BD Difco) containing 10 % (v/v) DMSO and stored at –80 °C.

This study presents the morphological, biochemical and molecular characterization of strains YIT 10443^T and YIT 10738. *Bifidobacterium minimum* YIT 4097^T and

Abbreviations: F6PPK, fructose-6-phosphate phosphoketolase; LAB, lactic acid bacteria; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains YIT 10443^T, YIT 10738, *B. minimum* YIT 4097^T and *B. psychraerophilum* YIT 11814^T are AB433856, AB433857, AB437350 and AB437351, respectively, and those for the *clpC* gene sequences of strains YIT 10443^T, YIT 10738, *B. minimum* YIT 4097^T and *B. psychraerophilum* YIT 11814^T are AB433858–AB433861, respectively.

RAPD-PCR fingerprints and scanning electron micrographs of the novel strains and a maximum-likelihood 16S rRNA gene sequence-based tree are available as supplementary material with the online version of this paper.

Bifidobacterium psychraerophilum YIT 11814^T were obtained from the culture collection of the Yakult Central Institute (YIT) and were used as reference strains. For further experiments, strains were cultivated and maintained in GAM broth (Nissui) supplemented with 1 % lactose (modified GAM broth, pH 6.8) at 30 °C for 1 or 2 days, unless indicated otherwise.

Chromosomal DNA used as a template for randomly amplified polymorphic DNA (RAPD) typing and 16S rRNA gene sequence amplification was prepared from the isolates according to the method of Watanabe *et al.* (2008). For discrimination of the isolates, RAPD fingerprinting was performed by a method described previously (Chao *et al.*, 2008). Primers A (5'-CCGCAGCCAA-3'), B (5'-AACGCGCAAC-3') and C (5'-GCGGAAATAG-3') were used. The two strains had different RAPD profiles (see Supplementary Fig. S1, available in IJSEM Online).

The partial 16S rRNA genes of strains YIT 10443^T and YIT 10738 were amplified by PCRs using universal primers BI8F (5'-GGGTTYCGATTCTGGCTCAGGATG-3') and 15R (5'-AAGGAGGTGATCCARCCGCA-3') (Miyake *et al.*, 1998). Partial ClpC ATPase (*clpC*) genes of the two strains were amplified using primers BClpC-F (5'-ATCGCSGARACBATYGAGA) and BClpC-R (5'-ATRATGCGCTTGTGCARYT-3'). These primers were designed from the consensus sequence of the *clpC* genes of *Bifidobacterium adolescentis* ATCC 15704 (GenBank accession no. AY722389), *B. animalis* subsp. *animalis* ATCC 25527^T (AY722380), *B. breve* UCC 2003 (AY722390), *B. dentium* JCM 1195^T (AY722387), *B. longum* subsp. *infantis* JCM 1222^T (AY722381), *B. pseudolongum* subsp. *globosum* JCM 5820^T (AY722386), *B. pullorum* LMG 21816 (AY722383) and *B. thermophilum* JCM 1207^T (AY722384). The PCR mixture (25 µl) contained 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 200 µM of each dNTP, 1 mM MgCl₂, 1 µg BSA, 0.5 U *Taq* DNA polymerase (Takara Bio Inc.), 0.1 µM each primer and 10 ng template DNA. The amplification program consisted of an initial heating step at 94 °C for 2 min, 30 cycles of 94 °C for 20 s, 55 °C for 30 s (for the 16S rRNA gene) or 58 °C for 20 s (for the *clpC* gene) and 72 °C for 20 s and a final extension step at 72 °C for 3 min. The PCR-amplified 16S rRNA and *clpC* genes from these strains were purified using a Montage PCR Filter unit (Millipore) according to the manufacturer's instructions. Cycle-sequencing reactions were performed using an ABI PRISM Big Dye Terminator v. 3.1 cycle-sequencing kit (Applied Biosystems) and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

16S rRNA gene sequences of strains YIT 10443^T and YIT 10738 were determined by the methods described previously (Chao *et al.*, 2008). The closest known relatives of the strains were determined by using FASTA, and sequences of closely related species were retrieved from DDBJ/GenBank/EMBL databases. Multiple alignment of the sequences was carried out with the CLUSTAL_X program

version 1.82 (Thompson *et al.*, 1997). Approximately 1450 bp of the 16S rRNA gene sequences of the strains and related species were used for constructing phylogenetic trees with the neighbour-joining method (Saitou & Nei, 1987). The statistical reliability of the tree was evaluated by bootstrap analysis of 1000 replicates (Felsenstein, 1985) and the tree topology was also confirmed with the maximum-parsimony (Fitch, 1971) and maximum-likelihood (Cavalli-Sforza & Edwards, 1967) methods, by using PHYLIP version 3.67 (Felsenstein, 2007). By using the EzTaxon program (Chun *et al.*, 2007), the similarities between the 16S rRNA gene sequences of YIT 10443^T and YIT 10738 was 99.9%. The sequence similarity between strain YIT 11443^T and its closest neighbours, *B. minimum* YIT 4097^T, *B. psychraerophilum* YIT 11814^T and *B. subtilis* DSM 20096^T, was 96.6, 95.7 and 95.8 %, respectively. The novel strains formed a subcluster with *B. minimum*, *B. psychraerophilum* and *B. subtilis* on the basis of neighbour-joining analysis (Fig. 1). Similar topologies were obtained by the maximum-parsimony (data not shown) and maximum-likelihood (Supplementary Fig. S2) methods. The similarities among *clpC* gene sequences (about 470 bp) of strains YIT 10443^T and YIT 10738 and their closest relatives *B. minimum* YIT 4097^T, *B. psychraerophilum* YIT 11814^T and *B. subtilis* LMG 11597^T ranged from 81.8 to 85.8 %. The similarity between the *clpC* gene sequences of YIT 10443^T and YIT 10738 was 99.4 %. Interspecies gaps within the genus *Bifidobacterium* based on *clpC* gene sequences normally exceed 10–15 % (81 % mean similarity; Ventura *et al.*, 2006), which suggested that these two strains are members of a novel *Bifidobacterium* species. On the basis of neighbour-joining analysis of *clpC* gene sequences, the novel strains did not belong to any existing recognized species and formed a distinct subcluster with *B. subtilis* (Fig. 2).

For determination of DNA–DNA relatedness and DNA G + C content, chromosomal DNA was extracted according to the method of Marmur (1961). DNA–DNA reassociation analyses were performed according to the microdilution well technique, using photobiotin for DNA labelling (Ezaki *et al.*, 1989). Reciprocal hybridization experiments were performed for each pair of strains at 50 °C for 15 min in the presence of 50 % formamide. DNA–DNA relatedness values between strains YIT 10443^T and YIT 10738 were 72–80 %, which showed that these two strains belonged to the same taxon, whereas the relatedness of YIT 10443^T to *B. minimum* YIT 4097^T and *B. psychraerophilum* YIT 11814^T was 11–15 % and 5–8 %, respectively. The hybridization values with the reference strains were well below the 70 % cut-off value that is recommended to separate species (Wayne *et al.*, 1987). To assess DNA base composition, DNA was degraded enzymically into nucleosides as described previously (Mesbah *et al.*, 1989) and then separated by HPLC. The DNA G + C contents of strains YIT 10443^T and YIT 10738 were 61.1 and 61.9 mol%.

Morphological, cultural and biochemical testing according to standard techniques was performed at 30 °C unless otherwise stated. Morphological characteristics of strains

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strains YIT 10443^T and YIT 10738 with closely related species. The tree was constructed by the neighbour-joining method on the basis of a comparison of approximately 1460 positions, and the sequence of *Escherichia coli* ATCC 11775^T was used as an outgroup. Bootstrap values (%) based on 1000 replications are given at nodes. Bar, 5 % sequence divergence.

Fig. 2. Phylogenetic tree based on *clpC* gene sequences showing the relationship of the novel strains isolated from airag with closely related species. The tree was constructed by the neighbour-joining method on the basis of a comparison of approximately 470 positions, and the sequence of *Bacillus subtilis* IS58 was used as an outgroup. Bootstrap values (%) based on 1000 replications are given at nodes. Bar, 10 % sequence divergence.

YIT 11443^T and YIT 11738 were determined by using a scanning electron microscope. The strains were cultured in modified GAM broth (supplemented with 1 % lactose) at 30 °C for 1–2 days under anaerobic conditions. After culturing, cells were washed with 0.05 M phosphate buffer (pH 7.0), fixed with 2.5 % glutaraldehyde for 1 h at 4 °C, post-fixed with 1 % osmium tetroxide for 1 h at 4 °C, dehydrated with a series of increasing ethanol concentrations (twice at 50, 70, 80, 90, 95 and 100 % for 20 min each) and soaked twice in 3-methyl butyl acetate for 20 min. The prepared cells were subsequently critical-point-dried in a critical point dryer (model HCP-2; Hitachi), sputter-coated with gold (model SC-701C-MC; SANYU Electron) and observed with a scanning electron microscope (model S-3400N; Hitachi). Scanning electron micrographs of cells of strains YIT 10443^T and YIT 10738 are shown in Supplementary Fig. S3. Gram staining and

catalase activity were determined by using cells grown on modified GAM agar at 30 °C for 2 days. Motility was tested in modified GAM soft agar (0.15 %). Carbohydrate fermentation tests were carried out by using the API 50CHL system (bioMérieux) in triplicate according to the manufacturer's instructions with some modification. Cells from agar plates were suspended in CHL broth supplemented with 0.025 % cysteine hydrochloride, inoculated onto the test strips and incubated in an anaerobic chamber at 30 °C for 5 days. F6PPK activity was determined according to the method described by Scardovi (1986). Cell-wall peptidoglycan was prepared and hydrolysed according to the method of Schleifer & Kandler (1972). Cell-wall amino acids were analysed by HPLC (Alliance 2695 HPLC system; Waters) equipped with a fluorescence detector (model 474; Waters) and an AccQ-Tag column (3.9 × 150 mm; Waters) and AccQ-Fluor reagent kit (6-

Table 1. Major differential characteristics between strains YIT 10443^T and YIT 10738 and genetically closely related bifidobacteria

Strains: 1, *Bifidobacterium mongoliense* sp. nov. YIT 10443^T; 2, *B. mongoliense* sp. nov. YIT 10738, 3, *B. minimum* YIT 4097^T; 4, *B. psychraerophilum* YIT 11814^T. Data were obtained in this study. All four strains produced acid from D-glucose, maltose, methyl α-D-glucopyranoside, turanose and sucrose. None of the strains produced acid from glycerol, erythritol, D-arabinose, D- or L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, N-acetylglucosamine, trehalose, inulin, xylitol, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol or 2- or 5-ketogluconate. +, Positive; –, negative; w, weakly positive.

Characteristics	1	2	3	4
Acid production from:				
L-Arabinose	+	+	–	+
D-Ribose	+	–	–	+
D-Xylose	–	–	–	+
D-Galactose	+	+	–	+
D-Fructose	–	w	+	+
D-Mannose	–	w	–	–
L-Sorbose	–	–	–	w
Amygdalin	+	–	–	+
Arbutin	w	–	–	+
Salicin	+	–	–	+
Cellobiose	+	–	–	+
Lactose	+	+	–	+
Melibiose	+	+	–	+
Melezitose	–	–	–	+
Raffinose	+	+	–	+
Starch	+	w	+	–
Glycogen	+	+	+	–
β-Gentiobiose	+	–	–	+
Gluconate	w	–	–	w
Aesculin hydrolysis	+	+	–	+
Growth at:				
15 °C	w	–	–	+
20 °C	+	+	w	+
35 °C	+	w	+	+
37 °C	+	–	+	+
40 °C	–	–	+	+
42 °C	–	–	w	–
Peptidoglycan type	L-Lys–D-Asp	L-Lys–D-Asp	L-Lys–L-Ser	L-Lys–L-Ser–D-Asp
DNA G + C content (mol%)	61.1	61.9	61.1	56.9

aminoquinolyl-*N*-hydroxysuccinimidylcarbamate; Waters) as the derivatization.

Various phenotypic characteristics of the novel strains differed from those of *B. minimum* YIT 4097^T and *B. psychraerophilum* YIT 11814^T, the phylogenetically closest related type strains (Table 1). According to the data obtained, the two strains are genetically distinguishable from recognized species of bifidobacteria and thus represent a novel species, for which we suggest the name *Bifidobacterium mongoliense* sp. nov.

Description of *Bifidobacterium mongoliense* sp. nov.

Bifidobacterium mongoliense (mon.go.li.en'se. N.L. neut. adj. *mongoliense* pertaining to Mongolia, from where the type strain was isolated).

Cells grown in modified GAM broth are rods of various shapes, 0.4–0.6 × 0.8–2 µm, with rounded or tapered ends, sometimes curved, swollen and branched. They are Gram-positive-staining, non-motile, asporogenous, F6PPK-positive, catalase- and oxidase-negative and facultatively anaerobic. Pinpoint colonies (0.2–0.4 mm in diameter) are formed under aerobic conditions after 2 days incubation at 30 °C. After anaerobic growth at 30 °C for 5–6 days, colonies on modified GAM agar are 2–3 mm in diameter; they are convex, white, opaque, smooth and circular with entire edges. The temperature range for growth is 15–35 °C; no growth occurs at 10 or 40 °C. The optimum temperature for growth is 25–30 °C. Grows at pH 4.5–8.0 with an optimum at pH 6.5–7.0. Acid is produced from L-arabinose, D-galactose, D-glucose, maltose, lactose, melibiose, raffinose, starch, glycogen, turanose, methyl α-D-glucopyranoside and sucrose. Acid is not produced from glycerol, erythritol, D-arabinose, D- or L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, N-acetylglucosamine, trehalose, inulin, melezitose, xylitol, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol or 2- or 5-ketogluconate. Acid production from D-ribose, amygdalin, arbutin, salicin, cellobiose, β-gentiobiose and gluconate is strain-dependent. Aesculin is hydrolysed. Peptidoglycan structure is L-Lys–D-Asp type with the presence of Lys, Glu, Ala and Asp. The DNA G + C content of the type strain is 61.1 mol%. Phylogenetic analysis of the 16S rRNA gene sequence places the species in the *B. minimum* subgroup of bifidobacteria.

The type strain, YIT 10443^T (=JCM 15461^T =DSM 21395^T), and the reference strain YIT 10738 were isolated from airag, a traditional fermented mare's milk product, which was collected in the Mongolian provinces of Umnugobi and Uburhangai, respectively, in 2004.

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