Bifidobacterium stercoris sp. nov., isolated from human faeces

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Strain Eg1^T, an anaerobic, Gram-stain-positive, non-motile and non-spore-forming bacterium, was isolated from human faeces. The optimal temperature for growth was 37 °C and tests for oxidase and catalase activities gave negative results. Fructose-6-phosphate phosphoketolase activity was detected. Acid was produced during fermentation of several substrates, including glucose. The end products of glucose fermentation were acetic acid and lactic acid, which were produced in a molar ratio of 1.76 : 1 (approximately 3 : 2). The G+C content was 57.8 mol%. Comparative analysis of 16S rRNA gene sequences showed that strain Eg1^T was closely related to *Bifidobacterium adolescentis* YIT 4011^T (98.36 % 16S rRNA gene sequence similarity) and *Bifidobacterium ruminantium* JCM 8222^T (97.93 %) and analysis of *hsp60* sequences showed that strain Eg1^T was closely related to *B. adolescentis* JCM 1275^T (99.35 % *hsp60* sequence similarity) and *B. ruminantium* JCM 8222^T (92.13 %). However, despite these degrees of similarity being high enough for strain Eg1^T to be included at the same species level as *B. adolescentis* and *B. ruminantium* (96.5–100 % for the genus *Bifidobacterium*), the isolate could be distinguished from *B. adolescentis* KCTC 3216^T and *B. ruminantium* KCTC 3425^T by low levels of DNA–DNA relatedness (41 and 17 %, respectively). Based on phenotypic, genotypic and phylogenetic analyses, we propose that strain Eg1^T is classified in a novel species, *Bifidobacterium stercoris* sp. nov. The type strain is Eg1^T (=KCTC 5756^T =JCM 15918^T).

Bifidobacteria are non-motile, non-sporulating, obligately anaerobic, non-gas-producing, saccharoclastic bacteria that belong to the family *Bifidobacteriaceae* in the class *Actinobacteria*, which comprises Gram-positive bacteria that have DNA with a high G+C content (Ventura *et al.*, 2007). As bifidobacteria facilitate the fermentation of a large variety of oligosaccharides, they are one of the most well-known probiotic bacteria and are considered the main target organism for inclusion in prebiotic foods, which may provide health benefits (Gibson & Roberfroid, 1995; Saulnier *et al.*, 2009; Ventura *et al.*, 2007). However, despite the physiological importance of bifidobacteria in the intestine, according to culture-independent studies (especially metagenomic studies based on the analysis of genomic DNA and rRNA gene libraries), they comprise only a small part of the diverse bacterial population found in the intestinal microbiota; however, the exact population in the human intestine has not been fully established (Eckburg *et al.*, 2005; Palmer *et al.*, 2007; Turroni *et al.*, 2009; Wang *et al.*, 2005). The general characteristics of the genus *Bifidobacterium* are the presence of fructose-6-phosphate phosphoketolase (F6PPK) and production of both acetic and lactic acids from glucose fermentation in a molar ratio of 3 : 2 (Scardovi, 1986). To date, 30 species and nine subspecies have been described as belonging to the genus *Bifidobacterium*.

The bifidobacterial strain Eg1^T was isolated from faeces of a healthy, 27-year-old Korean male on Eggerth–Gagnon agar supplemented with 5 % horse blood at 37 °C for 2 days in an anaerobic chamber (Bactron II; Sheldon Manufacturing) containing $N_2/H_2/CO_2$ (90 : 5 : 5). Strain Eg1^T was subcultured on *Bifidobacterium* medium (BM; DSMZ medium 58) under anaerobic conditions and stored as a suspension in 10 % skimmed milk (BBL) containing 10 % glycerol at −80 °C.

Bifidobacterium adolescentis KCTC 3216^T and *Bifidobacterium ruminantium* KCTC 3425^T were obtained from the Korean Collection for Type Cultures as reference strains. All experiments were performed using bacterial

Abbreviation: F6PPK, fructose-6-phosphate phosphoketolase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *hsp60* sequences of strain Eg1^T are FJ611793 and FJ770059, respectively.

A neighbour-joining phylogenetic tree based on partial *hsp60* sequences is available as supplementary material with the online version of this paper.

Table 1. Comparison of phenotypic characteristics of strain Eg1^T and its closest phylogenetic neighbours

Strains: 1, *Bifidobacterium stercoris* sp. nov. Eg1^T; 2, *B. adolescentis* KCTC 3216^T; 3, *B. ruminantium* KCTC 3425^T. Data were obtained in this study unless indicated. All strains were anaerobic and Gram-stain-positive, fermented glucose with production of acetic and lactic acids in a molar ratio of 3 : 2 and produced F6PPK (Biavati & Mattarelli, 1991; Gavini *et al.*, 1991; Scardovi, 1986) and were positive for production of acid from D-glucose, maltose, raffinose and salicin and production of acid phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, arginine arylamidase and proline arylamidase. All strains were negative for catalase, urease, gelatin hydrolysis, nitrate reduction, indole formation, D-mannose fermentation and production of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, α-fucosidase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, α-mannosidase, arginine dihydrolase, β-galactosidase-6-phosphate, glutamic acid decarboxylase, alanine arylamidase, cystine arylamidase, glutamyl glutamic acid arylamidase, leucyl glycine arylamidase, pyroglutamic acid arylamidase and valine arylamidase.

Characteristic	1	2	3
Aesculin hydrolysis	+	+	−
Acid production from:			
L-Arabinose	+	+	−
Cellobiose	+	−	−
D-Fructose	−	−	+
D-Galactose	−	+	+
Lactose	+	+	−
Melibiose	+	−	+
D-Ribose	+	−	+
Sucrose	−	−	+
Trehalose	+	−	−
Turanose	+	−	−
D-Xylose	+	+	−
D-Mannitol	+	+	−
D-Sorbitol	−	+	−
Methyl α-D-glucoside	+	−	−
Amygdalin	+	+	−
Arbutin	−	+	+
Glycogen	+	−	+
Starch	+	−	+
API Rapid ID 32A results			
α-Arabinosidase	−	+	−
α-Galactosidase	+	+	−
β-Galactosidase	+	+	−
α-Glucosidase	+	+	−
β-Glucosidase	+	+	−
Glycine arylamidase	+	+	−
Histidine arylamidase	+	+	−
Leucine arylamidase	+	+	−
Phenylalanine arylamidase	+	−	−
Serine arylamidase	+	−	−
Tyrosine arylamidase	+	−	−
API ZYM results			
α-Galactosidase	+	+	−
β-Galactosidase	+	+	−
α-Glucosidase	+	+	−
β-Glucosidase	+	+	−
DNA G+C content (mol%)*	57.8	59.0[a]	57.0[b]

Table 1. cont.

*Data obtained from: *a*, GenomesOnline (http://www.genomesonline.org/); *b*, Biavati & Mattarelli (1991).

strains cultivated and maintained in BM at pH 6.8 and 37 °C for 2 or 3 days unless stated otherwise. Gram staining was performed using a Gram-staining kit (bioMérieux). Spore staining was performed using malachite green dye. Phase-contrast microscopy (Nikon) was used to observe the morphology of individual cells as well as Gram and spore staining. To determine bile resistance, strain Eg1^T was cultivated in PYG medium (DSMZ medium 104) supplemented with 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 1.0 and 1.5 % (w/v) bile salts (Sigma). Catalase and oxidase activities were determined using 3 % (v/v) hydrogen peroxide and 1 % (w/v) *p*-tetramethylphenylenediamine solutions (bioMérieux), respectively. The motility of strain Eg1^T was determined by stabbing into PYG medium containing 0.4 % agar. Indole production and nitrate reduction were tested using indole nitrite medium (BBL). To investigate enzyme activities and acid production from various carbohydrates, API 50CHL, API 20A, API ZYM and Rapid ID 32A (bioMérieux) were used according to the manufacturer's instructions. F6PPK activity of strain Eg1^T was determined using a phosphoketolase assay (Orban & Patterson, 2000; Scardovi, 1986). The end products of glucose fermentation in cells cultured in *Lactobacillus* MRS broth (BBL) at 37 °C for 4 days were analysed by HPLC using an LC-10AT liquid chromatograph and an SPD-10A VP detector (Shimadzu) with an Alltech Prevail organic acid column (Grace Davison Discovery Sciences). The fermentation products were acetic acid and lactic acid in a molar ratio of 1.76 : 1, which closely corresponded to the usual molar ratio of 3 : 2 for acetic and lactic acid production by *Bifidobacterium* species (Scardovi, 1986). Other results of the phenotypic characterization are given in Table 1 and the species description.

To measure DNA–DNA relatedness and G+C content, genomic DNA was extracted from strain Eg1^T and the two reference strains using a G-spin Genomic DNA extraction kit (iNtRON Biotechnology). DNA–DNA relatedness was determined using the fluorometric method described by Ezaki *et al.* (1989) with some modifications (Hirayama *et al.*, 1996). Strain Eg1^T showed 41 % DNA–DNA relatedness to *B. adolescentis* KCTC 3216^T and 17 % DNA–DNA relatedness to *B. ruminantium* KCTC 3425^T. As reported by Wayne *et al.* (1987), a bacterial species should include strains with >70 % DNA–DNA relatedness, so the results of this study suggested that strain Eg1^T was a member of a novel species of the genus *Bifidobacterium*. The G+C

content of strain Eg1^T was determined using a fluorimetric method using SYBR Green I and real-time PCR (Gonzalez & Saiz-Jimenez, 2002). Genomic DNA from *Escherichia coli* K-12 and the reference strains was used for calibration (Gonzalez & Saiz-Jimenez, 2002). The G+C content of strain Eg1^T was estimated to be 57.8 mol%, which is consistent with the high G+C content (55–67 mol%) found in the family *Bifidobacteriaceae* (Jian *et al.*, 2001).

The 16S rRNA gene sequence of strain Eg1^T was amplified by colony PCR with four bacteria-specific primers (8F, 968F, 518R and 1492R; Baker *et al.*, 2003). The amplification products were purified using a QIAquick PCR Purification kit (Qiagen) and sequenced using a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems), according to the manufacturers' instructions. The reaction mixtures were analysed using an automated DNA analyser system (PRISM 3730XL DNA Analyzer; Applied Biosystems) and the 16S rRNA gene sequence fragments were assembled using SeqMan software (DNASTAR). The nearly full-length 16S rRNA gene sequence obtained from strain Eg1^T was compared with other sequences in the GenBank database. Strain Eg1^T was related to members of the genus *Bifidobacterium* in the phylum *Actinobacteria* and exhibited highest 16S rRNA gene sequence similarity to *B. adolescentis* YIT 4011^T and *B. ruminantium* JCM 8222^T (98.36 and 97.93 %, respectively). A total of 38 nearly full-length 16S rRNA gene sequences from reference strains were aligned with that of strain Eg1^T using the multiple sequence alignment program CLUSTAL X version 1.83 (Thompson *et al.*, 1997) and the alignment was trimmed and converted to MEGA format for phylogenetic analysis. Phylogenetic consensus trees using neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) algorithms were constructed and visualized using MEGA4 (Tamura *et al.*, 2007) and tested by randomly selecting 1000 bootstrap replicates. Phylogenetic consensus trees were visualized with MEGA4 (Tamura *et al.*, 2007). Phylogenetic analysis of 16S rRNA gene sequences showed that strain Eg1^T fell within the bifidobacteria cluster and was most closely related to *B. adolescentis* YIT 4011^T and *B. ruminantium* JCM 8222^T (Fig. 1).

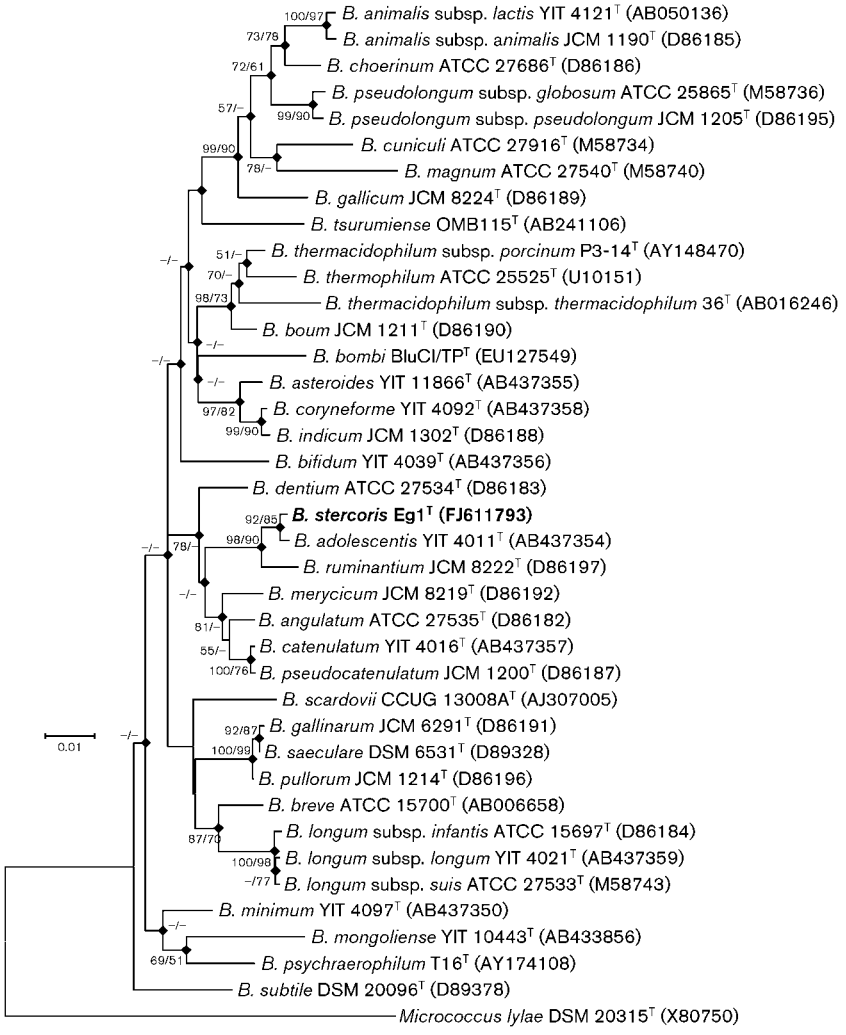

Fig. 1. Neighbour-joining phylogenetic consensus tree based on 16S rRNA gene sequences showing the phylogenetic position of strain Eg1^T in the genus *Bifidobacterium*. Filled diamonds indicate that the corresponding nodes were also recovered in the tree generated using the maximum-parsimony algorithm. Bootstrap values (>50 %) based on 1000 replicates for the neighbour-joining/maximum-parsimony methods are shown at branch nodes; –, bootstrap support ⩽50 %. Bar, 1 substitution per 100 nucleotide positions.

A housekeeping gene (*hsp60*, encoding the 60 kDa heat-shock protein) was used as an additional phylogenetic marker to identify bifidobacterial species (Jian *et al.*, 2001; Zhu *et al.*, 2003). The partial *hsp60* sequence of strain Eg1^T was obtained and analysed in a similar way to that for the 16S rRNA gene sequence, using the primers and method described by Okamoto *et al.* (2008) and the neighbour-joining algorithm. A 616-bp fragment of the *hsp60* sequence was obtained and analysed with 31 *hsp60* sequences from type strains of species of the genus *Bifidobacterium* (Supplementary Fig. S1, available in IJSEM Online). The *hsp60* sequence of strain Eg1^T showed 99.35 % sequence similarity to that of *B. adolescentis* JCM 1275^T, which indicated relatedness at the species level (96.5–100 %), and 92.13 % sequence similarity to that of *B. ruminantium* JCM 8222^T, indicating interspecies relatedness (80–96 %; Jian *et al.*, 2001; Zhu *et al.*, 2003). Even though analysis of the *hsp60* sequence is a more accurate tool for the identification of species of the genus *Bifidobacterium* than 16S rRNA gene sequence analysis, the low DNA–DNA relatedness (41 %) between strain Eg1^T and *B. adolescentis* KCTC 3216^T confirmed that these two strains belonged to different species.

On the basis of our phenotypic, genotypic and phylogenetic studies, we propose that strain Eg1^T is included in the genus *Bifidobacterium* as a member of a novel species, *Bifidobacterium stercoris* sp. nov.

Description of *Bifidobacterium stercoris* sp. nov.

Bifidobacterium stercoris (ster′co.ris. L. gen. n. *stercoris* of dung, excrement, ordure).

Anaerobic, Gram-stain-positive, non-motile and non-spore-forming. Cells are club-like or branch-shaped, 4.0–6.0 μm long and 1.0 μm wide, and are generally observed as single cells. Colonies on BM agar at 37 °C after 2 days are 1.0–1.5 mm in diameter, white and circular with a glistening surface, an umbonate side view and a mucoid texture. The optimal temperature for growth is 37 °C. Cells survive 0.05 and 0.1 % bile salts, but not 0.15 % bile salts. Oxidase- and catalase-negative. F6PPK activity is present. The end products of glucose fermentation are acetic acid and lactic acid in a molar ratio of 1.76 : 1 (about 3 : 2). Indole formation and nitrate reduction are negative. With API 20A, aesculin hydrolysis is positive, but gelatin hydrolysis and urease activity are negative. With API 50CHL, aesculin hydrolysis is positive and acid is produced from amygdalin, L-arabinose, cellobiose, D-glucose, methyl α-D-glucoside, glycogen, lactose, maltose, D-mannitol, melibiose, raffinose, D-ribose, salicin, starch, trehalose, turanose and D-xylose, but not from *N*-acetylglucosamine, D-adonitol, D-arabinose, D- or L-arabitol, arbutin, dulcitol, erythritol, D-fructose, D- or L-fucose, D-galactose, gentiobiose, gluconate, 2-ketogluconate, 5-ketogluconate, glycerol, inositol, inulin, D-lyxose, D-mannose, methyl α-D-mannoside, melezitose, L-rhamnose, D-sorbitol, L-sorbose, sucrose, D-tagatose, xylitol, L-xylose or methyl β-D-xyloside. With API Rapid ID 32A, positive for raffinose fermentation, α- and β-galactosidases, α- and β-glucosidases, arginine arylamidase, glycine arylamidase, histidine arylamidase, leucine arylamidase, phenylalanine arylamidase, proline arylamidase, serine arylamidase and tyrosine arylamidase, but negative for D-mannose fermentation, nitrate reduction, indole production, alkaline phosphatase, arginine dihydrolase, β-galactosidase-6-phosphate, α-arabinosidase, α-fucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, glutamic acid decarboxylase, alanine arylamidase, glutamyl glutamic acid arylamidase, leucyl glycine arylamidase, pyroglutamic acid arylamidase and urease. With API ZYM, positive for acid phosphatase, α- and β-galactosidases, α- and β-glucosidases, leucine arylamidase and naphthol-AS-BI-phosphohydrolase, but negative for alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, α-fucosidase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, α-mannosidase, cystine arylamidase and valine arylamidase. The G+C content of the type strain is 57.8 mol%.

The type strain, Eg1^T (=KCTC 5756^T =JCM 15918^T), was isolated from human faeces.

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