Bifidobacterium kashiwanohense sp. nov., isolated from healthy infant faeces

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Strains HM2-1 and HM2-2^T were isolated from the faeces of a healthy infant and were characterized by determining their phenotypic and biochemical features and phylogenetic positions based on partial 16S rRNA gene sequence analysis. They were Gram-positive, obligately anaerobic, non-spore-forming, non-gas-producing, and catalase-negative non-motile rods. They did not grow at 15 or 45 °C in anaerobic bacterial culture medium, and their DNA G + C content was in the range 56–59 mol%. In enzyme activity tests, strains HM2-1 and HM2-2^T were positive for α/β -galactosidases and α/β -glucosidases but negative for β -glucuronidase and cystine arylamidase. An analysis of the cell-wall composition of strains HM2-1 and HM2-2^T revealed the presence of glutamic acid, alanine and lysine. The presence of fructose-6-phosphate phosphoketolase shows that isolates HM2-1 and HM2-2^T are members of the genus *Bifidobacterium*. These two isolates belong to the same species of the genus *Bifidobacterium*. Strain HM2-2^T was found to be related to *Bifidobacterium catenulatum* JCM 1194^T (97.4% 16S rRNA gene sequence identity: 1480/1520 bp), *Bifidobacterium pseudocatenulatum* JCM 1200^T (97.2%: 1472/1514 bp), *Bifidobacterium dentium* ATCC 27534^T (96.7%: 1459/1509 bp) and *Bifidobacterium angulatum* ATCC 27535^T (96.5%: 1462/1515 bp). The predominant cellular fatty acids of strains HM2-1 and HM2-2^T were 16:0 and 18:1 ω 9c, with proportions greater than 18% of the total. Phylogenetic analyses involving phenotypic characterization, DNA–DNA hybridization and partial 16S rRNA gene sequencing proves that the strains represent a novel species of the genus *Bifidobacterium*, for which the name *Bifidobacterium kashiwanohense* sp. nov. is proposed. The type strain is HM2-2^T (=JCM 15439^T =DSM 21854^T).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and partial *hsp60* gene sequences of strain HM2-1 are AB491757 and AB578933, respectively. Those for strain HM2-2^T are AB425276.2 and AB491759.2, respectively.

Four supplementary figures are available with the online version of this paper.

The genus *Bifidobacterium* represents one of the bacterial groups within the class *Actinobacteria* and is prevalent within the gastrointestinal tracts of humans and animals (Mikkelsen *et al.*, 2003). At the time of writing, the genus *Bifidobacterium* is represented by over 30 species and subspecies, and the type strains of *Bifidobacterium adolescentis*, *B. angulatum*, *B. bifidum*, *B. breve*, *B. catenulatum*, *B. dentium*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, *B. pseudocatenulatum* and *B. scardovii* were isolated from human specimens (Scardovi, 1986; Hoyles *et al.*, 2002; Sakata *et al.*, 2002). The bifidobacteria have not been found to cause human diseases; instead, they are considered to be probiotic micro-organisms (Fuller, 1991; Ventura *et al.*, 2009; Whorwell *et al.*, 2006; Brenner *et al.*, 2009).

During our studies, two isolates, HM2-1 and HM2-2^T, from faeces of a healthy infant were not identified to the species level. Our results show that these isolates represent a novel *Bifidobacterium* species, for which the name *Bifidobacterium kashiwanohense* sp. nov. is proposed.

We isolated 51 strains of putative bifidobacteria inhabiting the faeces of a healthy infant (male; 1.5 years old) in 2008. Blood liver (BL) agar plates (Eiken Chemical) and anaerobic bacterial culture medium (ABCM broth; Eiken Chemical) were used for cell cultures. All 51 isolates were anaerobically cultured at 37 °C in ABCM broth and on BL agar plates for 24 and 72 h, respectively. Of the 51 isolates, *Bifidobacterium longum* subsp. *longum* (20 strains), *B. longum* subsp. *infantis* (2 strains), *B. bifidum* (6 strains) and *B. breve* (3 strains) were identified, whereas the other isolates (20 strains) were not identified to the species level. Of the 20 other isolates, the unidentified strains HM2-1 and HM2-2^T formed a subcluster in the genus *Bifidobacterium* in the taxonomic study. Strains HM2-1 and HM2-2^T were found to be Gram-positive, obligately anaerobic, non-spore-forming, non-gas-producing and catalase-negative non-motile rods. Table 1 shows the major characteristics of isolates HM2-1 and HM2-2^T, and the related strains *B. catenulatum* JCM 1194^T, *B. pseudocatenulatum* JCM 1200^T, *B. dentium* JCM 1195^T and *B. angulatum* JCM 7096^T. The results were recorded after 48 h at 37 °C. The isomer of lactic acid produced from D-glucose was determined by using an F-kit [D(-)-lactic acid/L(+) -lactic acid; Roche]. Other biochemical tests on motility, growth at a fixed temperature and gas production from D-glucose were performed using the methods described by Mitsuoka (1969). The optimal temperature for growth of strains HM2-1, HM2-2^T, JCM 7096^T and JCM 1194^T was 37 °C; no growth was observed at 15 or 45 °C in ABCM broth. Although all strains grew at pH 7.2 and pH 8.0, no growth was observed at pH 4.5. *B. pseudocatenulatum* JCM 1200^T grew weakly at 45 °C and pH 4.5. *B. dentium* JCM 1195^T grew at pH 4.5. Acid production from carbohydrates in ABCM broth is shown in Table 1. For the determination of phenotypic characteristics, API ZYM tests (bioMérieux) were performed in duplicate using the methods recommended by the manufacturer. Cells were collected from each ABCM culture by centrifugation at 12 000 r.p.m. for 5 min, inoculated onto the test strips and

Table 1. Physiological characteristics of isolates HM2-1 and HM2-2^T and the type strains of closely related species of the genus *Bifidobacterium*

Strains: 1, HM2-1; 2, HM2-2^T; 3, *B. catenulatum* JCM 1194^T; 4, *B. pseudocatenulatum* JCM 1200^T; 5, *B. dentium* JCM 1195^T; 6, *B. angulatum* JCM 7096^T. +, Positive; -, negative; w, weakly positive. All strains were positive for the following characteristics: fermentation of glucose, fructose, mannose, maltose and sucrose; growth in ABCM broth at 37 °C, at initial pH 7.2 and 8.0, and under anaerobic conditions; no growth in ABCM broth at 15 °C under anaerobic conditions. All strains produced L-lactic acid and did not produce gas from D-glucose. All strains produced acid from D-xylose, D-ribose, L-arabinose, D-glucose, D-galactose, sucrose, maltose, lactose, melibiose, raffinose and salicin. None of the strains produced acid from glycerol or rhamnose. All strains possessed leucine arylamidase, α -galactosidase, β -galactosidase, α -glucosidase and β -glucosidase; none possessed alkaline phosphatase, esterase (C4), lipase (C14), valine arylamidase, trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, N-acetyl- β -glucosaminidase, α -mannosidase or α -fucosidase. DNA G+C contents were determined by HPLC.

Characteristic	1	2	3	4	5	6
Growth at:						
45 °C	—	—	—	w	—	—
Initial pH 4.5	—	—	—	w	+	—
Acid from:						
D-Mannose	+	w	—	+	+	—
Cellobiose	+	+	+	+	+	—
Trehalose	—	—	—	+	+	—
Melezitose	—	—	—	—	w	—
Dextrin	w	w	w	+	+	+
Starch	—	—	—	+	+	+
Inulin	—	w	—	—	—	+
D-Mannitol	—	—	—	—	+	—
D-Sorbitol	+	+	+	+	—	—
D-Fructose	+	+	+	+	w	+
API ZYM						
Esterase lipase (C8)	+	+	+	—	—	—
Cystine arylamidase	—	—	+	—	—	—
Acid phosphatase	+	+	+	+	+	—
β -Glucuronidase	—	—	—	—	+	—
DNA G+C content (mol%)	58.6	56.3	54.7	57.5	61.2	59.0

incubated for 4 h at 37 °C. Nineteen enzymic activities were estimated by the API ZYM tests, and strains HM2-1 and HM2-2^T showed 7 enzymic activities (Table 1). Enzyme activity tests were positive for α/β -galactosidases and α/β -glucosidases but negative for β -glucuronidase and cystine arylamidase. Isolates HM2-1 and HM2-2^T had fructose-6-phosphate phosphoketolase activity like the reference strains (Biavati & Mattarelli, 1991; Gavini *et al.*, 1991). An analysis of the cell-wall composition revealed the presence of glutamic acid, alanine and lysine by ultraperformance liquid chromatography according to the methods described by Komagata & Suzuki (1987). The ACQUITY UPLC System (Waters) was used in this study. Whole-cell fatty acid methyl

esters were obtained by saponification, methylation and extraction followed by analysis using a standardized Microbial Identification System (Microbial ID) (Tan *et al.*, 2010). The Sherlock standard library MOORE5 5.00 made it possible to standardize the bacterial fatty acid analysis (<http://www.midi-inc.com>) in Table 2.

The DNA G+C contents of strains HM2-1 and HM2-2^T were determined in three separate trials by high-performance liquid chromatography (Okamoto *et al.*, 2008). The DNA G+C contents of strains HM2-1 and HM2-2^T were 58.6 and 56.3 mol%, respectively. These differ from the values obtained for *B. catenulatum* JCM 1194^T and *B. dentium* JCM 1195^T, whose DNA G+C contents were 54.7 and 61.2 mol%, respectively (Scardovi & Crociani, 1974). However, the DNA G+C content of strains HM2-1 and HM2-2^T were similar to those of *B. pseudocatenulatum* JCM 1200^T (57.5 mol%) and *B. angulatum* JCM 7096^T (59.0 mol%) (Scardovi & Crociani, 1974; Scardovi *et al.*, 1979).

The 16S rRNA genes of strains HM2-1 and HM2-2^T were amplified by PCR using the universal primers 27F and 1492R (Weisburg *et al.*, 1991). The closest known relatives of the isolate were determined by performing database searches, and the sequences of closely related species were retrieved from public databases. Multiple alignments of the sequences were carried out using CLUSTAL W (Thompson *et al.*, 1994). We used the neighbour-joining method to

reconstruct a phylogenetic tree (Saitou & Nei, 1987) and estimated the robustness of the individual branches by performing bootstrapping analysis with 1000 replicates (Felsenstein, 1985). Phylogenetic trees were also reconstructed using the maximum-likelihood (Cavalli-Sforza & Edwards, 1967) and maximum-parsimony (Kluge & Farris, 1969) methods with PHYLIP version 3.66. The partial 16S rRNA gene sequence of strain HM2-2^T was consistent with that of strain HM2-1. DNA–DNA hybridization was carried out using the microdilution-well technique, with photobiotin for DNA labelling (Ezaki *et al.*, 1989). Immobilized DNA in microdilution wells was incubated with a photobiotin-labelled DNA probe prepared from each strain at 48 °C for 3 h in hybridization buffer containing 50% formamide, after 30 min of incubation at 37 °C in prehybridization buffer. The fluorescence intensity was measured with a MicroPlate reader (PerkinElmer) at a wavelength of 360 nm for excitation and 450 nm for emission. Values (%) of DNA–DNA hybridization were the mean of the two trials in this study. Strains HM2-1 and HM2-2^T shared high levels of DNA–DNA relatedness (92.1%). We analysed the DNA profile by random amplified polymorphic DNA (RAPD) PCR performed using the random primers 103 (5'-GTGACGCCGC-3'), 127 (5'-ATCTGGCAGC-3') and 173 (5'-CAGGCGGCGT-3') (University of British Columbia) to compare strains HM2-1 and HM2-2^T. Their RAPD profiles (Supplementary Fig. S1 available in IJSEM Online) showed that they were different strains of the same species, supported by phenotypic and biochemical features and their phylogenetic position based on partial 16S rRNA gene sequence and DNA–DNA hybridization analyses.

Two organisms are considered to represent the same species if their purified DNA exhibits greater than 70% hybridization (Wayne *et al.*, 1987). DNA–DNA hybridization analysis was carried out comparing isolates HM2-1 and HM2-2^T and other related strains listed in Table 1 according to the method previously by Ezaki *et al.* (1989). DNA–DNA relatedness values between strain HM2-2^T and *B. catenulatum* JCM 1194^T were 8.1% ± 1.6% and 14.6% ± 2.4% using the probes for HM2-2^T and *B. catenulatum* JCM 1194^T, respectively. DNA–DNA relatedness values using the reference strains as DNA probes for strain HM2-2^T were as follows: HM2-2^T and *B. pseudocatenulatum* JCM 1200^T (6.5%), HM2-2^T and *B. dentium* JCM 1195^T (5.7%), and HM2-2^T and *B. angulatum* JCM 7096^T (6.8%).

We reconstructed a phylogenetic tree based on a total of 44 partial 16S rRNA gene sequences, including those of members of the genus *Bifidobacterium* and related genera. The tree was rooted using *Actinomyces bovis* (Fig. 1). Strains HM2-1 and HM2-2^T had 16S rRNA gene sequences that were similar to those of *B. catenulatum*, *B. angulatum* and *B. dentium*. Levels of similarity for the partial 16S rRNA gene sequence of HM2-2^T in relation to *B. catenulatum* JCM 1194^T, *B. pseudocatenulatum* JCM 1200^T, *B. dentium* ATCC 27534^T and *B. angulatum*

Table 2. Cellular fatty acid contents (%) of isolates HM2-1 and HM2-2^T and the type strains of closely related species of the genus *Bifidobacterium*

Strains: 1, HM2-1; 2, HM2-2^T; 3, *B. catenulatum*; 4, *B. pseudocatenulatum*; 5, *B. dentium*; 6, *B. angulatum*. Data for reference strains were obtained from the standard Library MOORE5 5.00 of the Sherlock Microbial Identification System.

Fatty acid	1	2	3	4	5	6
10:0	1.6	2.1	0.7	1.2	0.7	1.5
12:0	3.8	8.1	1.3	2.9	1.2	2.7
11:0 DMA	0.6	0.8	0.7	0.5	0	0
14:0	6.8	15.9	5.3	10.5	5.1	8.4
14:0 DMA	2.7	3.3	3.5	2.7	2.9	2.1
16:1 ω 9c	3.4	6.3	2.2	2.2	1.8	2.6
16:1 ω 7c	0	0	1.8	3.9	1.1	2.6
16:0	30.3	26.8	25.7	13.7	25.8	18.1
16:0 DMA	0.9	0.7	0.5	0	0	0
16:1 ω 7c DMA	0	0	1.1	0.5	0	0.6
17:0 anteiso	0	0	0	0.7	0	0
18:2 ω 6,9c	0.8	1.1	0	0	0	0
18:1 ω 9c	18.1	19.4	18.3	33.6	22.8	33.5
18:0	6.1	2.5	3.3	2.3	6.9	3.7
18:1 ω 7c DMA	0	0	0.6	0	0	0
18:0 12-OH	0	0	1.5	0	0.6	0
18:1 ω 9c DMA	19.1	9.1	23.3	12.2	23.2	14.3
18:0 DMA	0	0	0	0	0.6	0

Fig. 1. Phylogenetic relationship between isolates HM2-1 and HM2-2^T, species of the genus *Bifidobacterium* and related genera, determined by 16S rRNA gene sequencing. The tree was reconstructed using the neighbour-joining method. *Actinomyces bovis* NCTC 11535^T was used as an outgroup. Bootstrap percentages (based on 1000 replicates) are shown at nodes; values lower than 50% are not indicated. Accession numbers are given in parentheses. Bar, 10% difference in nucleotide sequence.

ATCC 27535^T were 97.4% (1480/1520 bp), 97.2% (1472/1514 bp), 96.7% (1459/1509 bp) and 96.5% (1462/1515 bp), respectively. We obtained identical tree topologies in the maximum-parsimony and maximum-likelihood analyses (Supplementary Figs S2 and S3).

Heat-shock protein (*hsp60*; also known as *groEL*, *cpn60*, *groES* or *dnaK*) genes have also been adopted for taxonomic studies of bifidobacterial species (Jian *et al.*, 2001; Zhu *et al.*, 2003; Simpson *et al.*, 2004; Delcenserie *et al.*, 2005; Ventura *et al.*, 2005). The partial *hsp60* gene sequence of strain HM2-2^T was obtained by using the conditions and primers for PCR amplification described by Jian *et al.* (2001) and Okamoto *et al.* (2008). We reconstructed a phylogenetic tree

based on a total of 40 sequences of *hsp60* genes, including those of members of the genus *Bifidobacterium* and related genera (Supplementary Fig. S4). Levels of similarity for the partial *hsp60* gene sequence of HM2-2^T in relation to *B. catenulatum* JCM 1194^T, *B. pseudocatenulatum* JCM 1200^T, *B. dentium* ATCC 27534^T and *B. angulatum* ATCC 27535^T were 96.1% (567/590 bp), 90.8% (536/590 bp), 92.0% (544/591 bp) and 89.8% (531/591 bp), respectively. The 16S rRNA and *hsp60* gene sequences were well related and useful for determining the phylogeny of strain HM2-2^T.

Detailed characteristics of the novel isolates are provided in the species description and in Table 1, and these characteristics were compared with those of the phylogenetic relatives

B. catenulatum JCM 1194^T, *B. pseudocatenulatum* JCM 1200^T, *B. angulatum* JCM 7096^T and *B. dentium* JCM 1195^T. The isolates were able to produce L(+) -lactic acid from D-glucose. Anaerobic conditions were essential for the growth of strains HM2-1 and HM2-2^T. These two isolates could be distinguished from *B. pseudocatenulatum* JCM 1200^T, *B. angulatum* JCM 7096^T and *B. dentium* JCM 1195^T by the pattern of acid production from D-mannose, cellobiose, trehalose, melezitose, dextrin, starch, inulin, D-mannitol, D-sorbitol and D-fructose. The cellular fatty acid profiles of strains HM2-1, HM2-2^T and the related genera *B. catenulatum*, *B. pseudocatenulatum*, *B. dentium* and *B. angulatum* are presented in Table 2. The predominant cellular fatty acids of strains HM2-1 and HM2-2^T were 16:0 and 18:1ω9c, with proportions greater than 18 % of the total.

Experimental data suggest that isolates HM2-1 and HM2-2^T can be assigned to the genus *Bifidobacterium* on the basis of their phylogenetic position; it can be concluded that these two isolates might be considered as representing a single novel species. Thus, these two isolates represent a novel species, for which we propose the name *Bifidobacterium kashiwanohense* sp. nov.

Description of *Bifidobacterium kashiwanohense* sp. nov.

Bifidobacterium kashiwanohense (ka.shi.wa.no.hen'se. N.L. neut. adj. *kashiwanohense* of Kashiwanoha in Japan, which is the name of the area, University of Tokyo, where this bacterium was originally isolated).

Cells are Gram-positive, non-motile, non-spore-forming rods measuring 1.0 × 1.3 µm. Colonies on BL agar after incubation under anaerobic conditions for 2 days at 37 °C are beige, smooth and approximately 1.0 mm in diameter. No growth is observed at 15 or 45 °C. Produces L(+) -lactic acid from D-glucose. Produces acid from D-xylose, D-ribose, D-mannose, cellobiose, D-sorbitol, L-arabinose, D-glucose, D-fructose, D-galactose, sucrose, maltose, lactose, melibiose, raffinose and salicin, and, to a weaker extent, from dextrin. Exhibits esterase lipase (C8), leucine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, and fructose-6-phosphate phosphoketolase activities. The cell wall contains glutamic acid, alanine and lysine. The DNA G + C content is 56–59 mol%.

The type strain is HM2-2^T (=JCM 15439^T =DSM 21854^T), isolated from faeces of a healthy infant (1.5 years old).

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