***Bifidobacterium thermacidophilum* sp. nov., isolated from an anaerobic digester**

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A new phenotypic group of *Bifidobacterium* strains, isolated from an anaerobic digester for the treatment of waste water from a bean-curd farm, was described previously. In this study, the DNA–DNA relatedness between strain 36 (type strain, AS 1.2282^T) of this new group and the type strains of other described *Bifidobacterium* species was analysed. The low level of DNA homology (0–58.9%) as well as comparison of the 16S rDNA sequences confirmed the distinct phylogenetic position of strain 36. In addition, the new species differed from other *Bifidobacterium* species in its phenotypic characteristics, such as its growth at moderately thermophilic conditions (49.5 °C) and at relatively low pH (4.0), as well as its sugar-fermentation pattern. On the basis of phenotypic, genetic and phylogenetic studies, a new *Bifidobacterium* species, *Bifidobacterium thermacidophilum* sp. nov., was designated.

Keywords: anaerobic digester, *Bifidobacterium thermacidophilum*, DNA relatedness, phylogeny

INTRODUCTION

Since *Bifidobacterium* was first described by Tissier in 1899, 32 species have been assigned to this genus (Scardovi, 1986; Biavati *et al.*, 1991; Biavati & Mattarelli, 1991; Watabe *et al.*, 1983; Crociani *et al.*, 1996; Meile *et al.*, 1997; Lauer, 1990). The bacteria in this group were mainly isolated from the intestines of humans and animals. They were believed to be predominantly a constituent of the intestinal flora. Thus far, no *Bifidobacterium* strains have been found in clinical specimens, except for three species found in human dental caries. In recent years, these bacteria have been used as probiotics to suppress intestinal pathogens. In addition, bifidobacteria have also been found in other anoxic environments, such as sewage. Recently, *Bifidobacterium lactis* was isolated from fermented milk (Meile *et al.*, 1997).

Though some of the species of *Bifidobacterium* showed phenotypic and genetic heterogeneity (Chevalier *et al.*, 1990), the validity of most species was confirmed by 16S rRNA sequence homology analyses (Leblond-Bourget *et al.*, 1996; Stackebrandt & Goebel, 1994). Furthermore, most members of the genus *Bifido-*

bacterium were demonstrated, by DNA–DNA relatedness determinations (Lauer & Kandler, 1983; Stackebrandt & Goebel, 1994), to be the well-established species.

In a previous study in our laboratory, 42 strains of bifidobacteria were isolated from human and animal faeces and from an anaerobic digester for the treatment of waste water from a bean-curd farm in Beijing (Ling *et al.*, 1996). The assignment of strains to the genus *Bifidobacterium* was done on the basis of the following diagnostic characteristics: they were Gram-positive, pleomorphic, irregular, non-spore-forming rods; they showed anaerobic growth; they were non-motile and catalase-negative; and they produced acetic and lactic acid as the main products of glucose fermentation and did not produce gas. A total of six phenotypic clusters were defined from numerical analysis based on 75 features. Strains in cluster E, all of which were isolated from the waste water of the bean-curd farm, clustered distantly from other strains, with a quite low S_M value (< 60%). In addition, this group of strains could not be identified as one of the existing *Bifidobacterium* species according to the conventional classification. To clarify their taxonomic position, the DNA–DNA relatedness between one of the new isolates and existing members of the genus *Bifidobacterium* was determined. The results revealed a new species of the

The GenBank/EMBL/DBJ accession number for the 16S rDNA sequence of strain 36 reported in this paper is AB016246.

genus *Bifidobacterium*. Its phylogenetic relationship with other species was also analysed according to 16S rDNA sequence homologies.

METHODS

Bacterial strains. All of the bifidobacterial strains used in this study are listed in Table 1. The reference strains were kindly provided by the Japan Collection of Microorganisms (JCM) and the German Collection of Microorganisms and Cell Cultures (DSMZ), respectively. The strains from our collection were isolated by the Hungate anaerobic roll-tube technique and were routinely grown in tryptone peptone yeast extract glucose (TPYG) medium (Scardovi, 1986). The

media were prepared in glass tubes sealed with butyl rubber stoppers and screw caps. The gas phase was N₂. After inoculation, tubes were incubated at 37 °C. The cultures for the tests below were all cultivated anaerobically unless otherwise indicated.

Characterization of the strains. Fructose-6-phosphate phosphoketolase activity in cell extract was assayed as described previously (Scardovi, 1986).

End products from glucose fermentation were detected by GC (GC-7AG; Shimadzu). Bacterial strains were inoculated in TPYG media as well as in tryptone peptone yeast extract (TPY) media without glucose (as the control). After 48 h growth, the products were analysed. The acids produced during glucose fermentation and the ratio of acetic acid to

Table 1. DNA homologies between *Bifidobacterium thermacidophilum* 36^T and existing species of the genus *Bifidobacterium*

<i>Bifidobacterium</i> species	Reassociation with DNA of <i>B. thermacidophilum</i> 36 ^T (%)
<i>B. thermacidophilum</i> 36 ^T (AS 1.2282 ^T)	100.0*
<i>B. thermacidophilum</i> 35	87.0, 92.1, 94.0*
<i>B. thermacidophilum</i> 37	92.5, 94.3, 118.6*
<i>B. adolescentis</i> JCM 1275 ^T (ATCC 15703 ^T)	17.9
<i>B. angulatum</i> JCM 7096 ^T (ATCC 27535 ^T)	38.7
<i>B. animalis</i> JCM 1190 ^T (ATCC 25527 ^T)	28.5
<i>B. asteroides</i> JCM 8230 ^T (ATCC 25910 ^T)	29.5
<i>B. bifidum</i> JCM 1255 ^T (ATCC 29521 ^T)	34.7
<i>B. boum</i> JCM 1211 ^T (ATCC 27917 ^T)	34.3
<i>B. breve</i> JCM 1192 ^T (ATCC 15700 ^T)	9.0
<i>B. catenulatum</i> JCM 1194 ^T (ATCC 27539 ^T)	22.9
<i>B. choerinum</i> JCM 1212 ^T (ATCC 27686 ^T)	24.6
<i>B. coryneforme</i> JCM 5819 ^T (ATCC 25911 ^T)	24.0
<i>B. cuniculi</i> JCM 1213 ^T (ATCC 27916 ^T)	41.4
<i>B. dentium</i> JCM 1195 ^T (ATCC 27534 ^T)	42.0
<i>B. denticolens</i> DSM 10105 ^T	47.3
<i>B. gallicum</i> JCM 8224 ^T (ATCC 49850 ^T)	26.3
<i>B. gallinarum</i> JCM 6291 ^T (ATCC 33777 ^T)	34.3
<i>B. globosum</i> JCM 5820 ^T (ATCC 25865 ^T)	36.8
<i>B. indicum</i> JCM 1302 ^T (ATCC 25912 ^T)	31.6
<i>B. inopinatum</i> DSM 10107 ^T	0
<i>B. infantis</i> JCM 1222 ^T (ATCC 15697 ^T)	26.0
<i>B. lactis</i> DSM 10140 ^T	34.6
<i>B. longum</i> JCM 1217 ^T (ATCC 15707 ^T)	27.7
<i>B. magnum</i> JCM 1218 ^T (ATCC 27540 ^T)	0
<i>B. merycicum</i> JCM 8219 ^T (ATCC 49391 ^T)	25.0
<i>B. minimum</i> JCM 5821 ^T (ATCC 27538 ^T)	22.9
<i>B. pseudocatenulatum</i> JCM 1200 ^T (ATCC 27910 ^T)	30.9
<i>B. pseudolongum</i> JCM 1205 ^T (ATCC 25526 ^T)	30.5
<i>B. pullorum</i> JCM 1214 ^T (ATCC 27685 ^T)	24.5
<i>B. ruminantium</i> JCM 8222 ^T (ATCC 49390 ^T)	31.6
<i>B. saeculare</i> JCM 8223 ^T (ATCC 49392 ^T)	27.8
<i>B. subtile</i> JCM 5822 ^T (ATCC 27535 ^T)	19.3
<i>B. suis</i> JCM 1269 ^T (ATCC 27533 ^T)	3.0
<i>B. thermophilum</i> JCM 1207 ^T (ATCC 25525 ^T)	58.9

* DNA reassociation rates (%) among the three strains of *B. thermacidophilum* refer to the determinations using three different batches of DNA.

lactic acid were measured according to the methods described by Holdeman *et al.* (1977).

The determination of sugar-fermentation patterns was achieved as follows. Sugars (final concentration 1%) sterilized by filtration were added aseptically to the autoclaved TPY broth (pH 7.0–7.2) without substrate. The TPY/sugar broths were inoculated with the different strains and cultivated at 37 °C for 48 h or longer, and the decrease in the pH value was followed by using bromothymol blue (0.04%) and methyl red (0.02%) as pH indicators. The temperature profiles were determined using a water bath with a temperature gradient. Initial pH values and the NaCl concentrations of the media were obtained by adding HCl (0.2 M) and the required amount of NaCl to TPYG broth, respectively. Growth was measured using a spectrophotometer at a wavelength of 600 nm after cultivation at 37 °C for 24 h to 1 week. All of the tests were performed in duplicate.

DNA extraction. The chromosomal DNA was extracted using a modified version of the method of Marmur (1961). As the cell walls of bifidobacteria were hard to lyse with lysozyme and SDS, 0.5 mg penicillin G ml⁻¹ culture was added to the cultures at late exponential phase to assist cell breakage. The cultures were incubated for another 16–20 h. To obtain higher yields of DNA, protease K (100 µg ml⁻¹, 37 °C for 1.5–2 h) was used instead of phenol, to remove the contaminated protein.

Determination of G + C content of DNA and DNA relatedness analysis. The G + C content of the DNA was determined by thermal denaturation (Marmur & Doty, 1962). The DNA from *Escherichia coli* K-12 was used as reference for thermal melting profile (T_m) determinations. DNA–DNA relatedness between the new isolates and all the reference strains was determined on the basis of the DNA–DNA liquid reassociation rate, as described by De Ley *et al.* (1970). The purity and concentration of each DNA preparation in 0.1 × SSC (saline sodium citrate buffer) were determined by measuring the optical density at 260 nm. The amount of DNA used to determine the reassociation rate was approximately 75–80 µg ml⁻¹. After the DNA was sheared to approximately 2.5×10^6 Da by passage through a syringe with a narrow needle (No. 5), the preparation buffer was adjusted to 2 × SSC to determine the DNA–DNA reassociation rate (v'). The determinations were all performed at 73 °C using a 752 Spectrophotometer with a thermometer controller (Shanghai 3rd Analytic Factory) and the renaturation was followed for 30 min. Normally, three batches of DNA per strain were used; only v' values of $\geq 3.0 \times 10^{-3}$ min⁻¹ for self-renaturation were used for DNA–DNA relatedness analysis.

Amplification and cloning of 16S rDNA. Chromosomal DNA prepared as described above was used as the template for PCR amplification; the two primers, 27f (5'-AGAGTTTG-ATCC/ATGGCTCAG-3') and 1541R (5'-AAGGAGGT-GATCCAGCC-3'), were complementary to positions 8–27 and 1525–1541 of 16S rDNA, respectively. Each PCR mixture (50 µl) contained the following: approximately 300 ng DNA template; 100 µmol each of dATP, dCTP, dGTP and dTTP; 2.5 mM Mg²⁺; 40 pmol each of the two primers; and 2.5 U *Taq* DNA polymerase. The following thermal cycling conditions were used: initial denaturing of DNA template at 94 °C for 5 min followed by 30 cycles comprising (1) denaturation at 94 °C for 1 min, (2) annealing at 52 °C for 1 min and (3) elongation at 72 °C for 3 min. When the 30 cycles were complete, the reaction mixture was

maintained at 72 °C for 2–3 h. The PCR amplification was performed with a Thermolyne Amplitron I (Barnstead Thermolyne Corporation).

PCR products were purified by agarose gel electrophoresis and electroelution, as described previously (Sambrook *et al.*, 1989), and then ligated to a plasmid pSK-T vector as described by Harrison *et al.* (1994). The recombinant plasmids with the 16S rDNA insertion were transformed into *E. coli* DH5 α .

16S rDNA sequencing. Plasmid pSK-T containing the 16S rDNA fragment was extracted and purified by polyethylene glycol precipitation according to the method described previously (Sambrook *et al.*, 1989). Nucleotide sequencing was performed using the Sanger dideoxynucleotide chain-termination method. The complete 16S rDNA sequence was determined by using a Dye Terminator Cycle-sequencing Ready Reaction kit (Perkin Elmer). Sequencing was performed with Applied Biosystems 373A-18 equipment. The following oligonucleotides were used as the sequencing primers: the T7 promoter (5'-TAATACGACTCACTATA-GGG-3'), the T3 promoter (5'-ATTAACCCTCACTAAA-GGGA-3'), 704F (5'-GTAACGGTGGGAATGTGTAGA-3') and 765R (5'-CCTGTTCGCTCCCCACGCTT-3'). The latter two were complementary to positions 685–704 and 765–784 of the 16S rDNA, respectively.

Analysis of sequence data and phylogenetic tree construction. The 16S rDNA sequence determined and those of the reference strains obtained from EMBL/GenBank/DDBI were aligned by CLUSTAL W software (version 1.5) and the similarities were calculated and converted to a distance matrix with the Jukes–Cantor coefficient by the DNADIST program (PHYLIP, version 3.5; Felsenstein, 1993). A dendrogram was produced by the Fitch method (PHYLIP, version 3.5) and the topology of the trees was evaluated by bootstrap analysis of the sequence data with the SEQUENCEBOOTS program (PHYLIP version 3.5).

RESULTS AND DISCUSSION

The members of the new group of *Bifidobacterium* strains in this study produced acetic and lactic acid in a molar ratio of 2.46–2.72:1 during glucose fermentation. Cells contained fructose-6-phosphate phosphoketolase activities when growing in TPYG broth. Furthermore, their chromosomal DNA, when used as templates for a PCR reaction, as described by Kaufmann *et al.* (1997), allowed a *Bifidobacterium* genus-specific 16S rDNA fragment (1.35 kb) to be amplified (data not shown). Thus, their membership of the genus *Bifidobacterium* was further confirmed by these analyses, as well as by the characteristics published previously (Ling *et al.*, 1996).

Morphology

Cells were pleomorphic, rod-, club- or 'V'-shaped when grown in TPYG media, and 0.5×1.5 – 2.5 µm in size in 24 h cultures (Fig. 1). The cell wall showed typical Gram-positive staining. Although aggregated cell clumps were formed in liquid cultures, cells were readily dispersed by shaking. Colonies on TPYG agar were convex, entire, white and 2 mm in diameter after 24 h cultivation.

Fig. 1. Micrograph of *Bifidobacterium thermacidophilum* sp. nov. AS 1.2282^T grown on a TYPG slant for 18 h. Bar, 5 μm.

Physiological features

The strains shared the common characteristics of *Bifidobacterium* species, except for the following features. All three strains grew in media with an initial

pH of 4.0 and at temperatures up to 48.5 °C (strains 36 and 37 even grew at 49.5 °C). *Bifidobacterium thermophilum* JCM1207^T grew quite poorly at 48.5 °C and did not grow at all at 49.5 °C. *Bifidobacterium boum* JCM 1211^T grew at an even lower temperature (≤ 45 °C) and at a more alkaline pH (≥ 5.0) (Scardovi *et al.*, 1979). All of the strains grew very well in a medium containing 2.0% but not 2.5% (w/v) NaCl.

Table 2 shows the carbohydrate fermentation by the three strains of the new species. Two out of the three strains did not ferment lactose but fermented some pentoses and oligo- or polysaccharides such as raffinose, melezitose and starch. These characteristics were rarely shared with most of the other *Bifidobacterium* species.

G + C content of the DNA

The G + C content of the DNA of the new species was determined to be 56.85 ± 2.05 mol% and that of the type strain, AS 1.2282^T, was 57.7 mol%.

DNA–DNA relatedness

DNA–DNA relatedness was determined using DNA–DNA liquid reassociation rates. The levels of DNA–DNA relatedness among the new strains as well as those between the new species and all of the existing *Bifidobacterium* species are listed in Table 1. The high

Table 2. Differential characteristics of *Bifidobacterium thermacidophilum* sp. nov., *Bifidobacterium thermophilum* and *Bifidobacterium boum*

+, Positive reaction; –, negative reaction; w, weak reaction; d, reaction variable among strains. All strains were positive for acid from D-fructose, D-galactose, melibiose, sucrose and raffinose. All strains were negative for acid from D-mannose and D-mannitol.

Character	<i>B. thermacidophilum</i>			<i>B. thermophilum</i> JCM 1207 ^T	<i>B. boum</i> [*]
	35	36	37		
Acid from:					
D-Gluconate	–	w	w	–	–
D-Ribose	+	–	w	–	–
L-Arabinose	+	+	w	–	–
D-Xylose	+	–	–	–	–
D-Rhamnose	–	–	–		
D-Trehalose	–	–	–	d	–
D-Sorbitol	+	+	+	–	–
D-Sorbose	–	–	–		
Lactose	+	–	–	d	d
Melezitose	–	+	+	d	–
Inulin	–	–	–	d	+
Starch	–	+	+	+	+
Salicin	+	–	–	d	–
Growth at:					
Maximum temp. (°C)	48.5	49.5	49.5	47.5	45
Minimum pH	4.0	4.0	4.0	4.2	> 5.0
G + C content (mol %)	55.4	57.7	58.3	60.0	60.0

* From Scardovi *et al.* (1979).

Table 3. Comparison of percentage DNA relatedness levels between some known *Bifidobacterium* species, determined using DNA reassociation rates and membrane hybridization

Species tested	DNA reassociation rate (%) [*]	Membrane hybridization (%) [†]
<i>B. pseudolongum</i> JCM 1205 ^T × <i>B. globosum</i> JCM 5820 ^T	78.4	76.0–80.0
<i>B. dentium</i> JCM 1195 ^T × <i>B. adolescentis</i> JCM 1275 ^T	61.2	50.0–59.0
<i>B. adolescentis</i> JCM 1275 ^T × <i>B. infantis</i> JCM 1222 ^T	34.1	36.0
<i>B. infantis</i> JCM 1222 ^T × <i>B. thermophilum</i> JCM 1207 ^T	38.0	23.0
<i>B. infantis</i> JCM 1222 ^T × <i>B. infantis</i> JCM 1220	101.9	—

^{*} Results from this study.

[†] Results from Lauer & Kandler (1983).

DNA homologies (ranging from 87.0 to 108 %) within the new group indicated that they were a homogeneous genetic group, although strain 35 was a little distant. However, the levels of DNA relatedness between the new strain and all of the existing *Bifidobacterium* species ranged from 0 to 58.9 %. This is obviously lower than the species-defining border (70 % DNA homology), hence a new species of *Bifidobacterium* should be defined. The highest DNA reassociation value was measured between the new strain and *B. thermophilum*, indicating a closer relationship between these two strains. A possible explanation for this is their shared thermophilic characteristic.

To test the reliability of the liquid reassociation-rate method we used, DNA–DNA homologies between some of the reference strains were measured. Table 3 shows that the DNA–DNA reassociation rates obtained by us were in accordance with those obtained by membrane hybridization (Lauer & Kandler, 1983).

Phylogenetic position of *Bifidobacterium thermacidophilum* sp. nov.

The complete 16S rDNA of *Bifidobacterium thermacidophilum* sp. nov. (1533 bp) was sequenced and compared with sequences from 20 type species of *Bifidobacterium* and that of the type strain of *Gardnerella vaginalis*. On the basis of the 16S rDNA sequences in a consensus length of 1438 bp, the similarity levels were analysed and a phylogenetic tree rooted with *G. vaginalis* was constructed (Fig. 2). The results indicated that *B. thermacidophilum* was relatively close to *B. thermophilum* (92.70 % similarity), and both formed a distinct phylum relative to other *Bifidobacterium* species.

As the new species was related to *B. thermophilum*, both phylogenetically and genetically, some of their phenotypic characteristics were compared. According to Scardovi (1986), *B. thermophilum* and *B. boum* were closely related, so the latter species was also included for comparison. Table 2 shows that *B. thermacidophilum* fermented pentoses such as arabinose, ribose and sorbitol, unlike *B. thermophilum* and *B. boum*. The

Fig. 2. Dendrogram based on 16S rDNA sequences in a consensus length of 1438 bp, indicating the phylogenetic relationship of *Bifidobacterium thermacidophilum* 36 and 20 existing *Bifidobacterium* spp. The tree was rooted with *Gardnerella vaginalis* and constructed by using the Fitch method. The bootstrap values were calculated from 100 trees. The number at each branch point represents percentage bootstrap support. Bar, 1 % sequence divergence.

new species also survived better in unfavourable environmental conditions such as elevated temperatures as high as 49.5 °C and low pH.

The phylogenetic analysis in this study showed unreasonably high similarities (≥ 97.7 %) between the 16S rRNA sequences of *Bifidobacterium pseudolongum* ATCC 25526^T and *Bifidobacterium longum* ATCC 15707^T and those of *Bifidobacterium indicum* ATCC 25912^T and *Bifidobacterium infantis* ATCC 15697^T, respectively. However, the two pairs of species were demonstrated to be distantly related by DNA–DNA relatedness (Lauer & Kandler, 1983). A recent study (Miyake *et al.*, 1998) indicated that the disparity between phylogenetic and DNA–DNA relatedness analyses of these strains was caused by the incorrectly

registered sequences of *B. pseudolongum* ATCC 25526^T (M58742) and *B. indicum* ATCC 25912^T (M58737) in the databases. Phylogenetic analysis based on the registered sequences (D86187 and D86188) of the two strains showed that *B. pseudolongum* ATCC 25526^T and *B. indicum* ATCC 25912^T were close to *Bifidobacterium globosum* and *Bifidobacterium coryneforme*, respectively. Because the new sequences have not appeared in EMBL/GenBank/DBJ thus far, the two old sequences had to be used for phylogenetic tree construction in this study.

Description of *Bifidobacterium thermacidophilum* sp. nov.

Bifidobacterium thermacidophilum (therm.ac.id.o'phil.um. Gr. n. *therme* heat; M.L. n. *acidum* acid; Gr. adj. *philus* loving; M.L. adj. *thermacidophilum* heat/acid-loving).

Gram-positive, non-motile, non-sporing irregular rods, arranged singly or occasionally in a 'V' shape, are 0.5 × 1.5–2.5 µm in size after 16–24 h in TPYG liquid at 37 °C. Liquid cultures of some strains can form a homogenous sediment, but it is readily dispersed by shaking. Anaerobic growth also occurs in standing culture under 90% air plus 10% CO₂. Colonies on TPYG agar are circular, convex with entire edges, white and reach 1.5–2 mm in diameter after 24 h cultivation. The optimum temperature for growth is 37–41 °C while the temperature range for growth is 30–49.5 °C. The optimum initial pH is 7.0–7.2; growth at pH 4.5 is quite good but is delayed at pH 4.0. Fructose, galactose, arabinose, sorbitol, melibiose, sucrose, raffinose, melezitose and starch are fermented. Mannose, mannitol, rhamnose, trehalose, sorbose and inulin are not fermented. Fermentation of gluconate, ribose, xylose, lactose and salicin is variable. Catalase is negative. Gelatin is not liquefied. H₂S is not produced. Litmus milk is not acidified and coagulated by most of the strains. The fermentation products from glucose are acetic and lactic acid at a molar ratio of 2.46–2.72:1. Final pH values of 3.6 can be reached. The G + C content of the DNA is 56.85 ± 2.05 mol% (57.7 mol% for the type strain). The micro-organism was found in the waste water of a bean-curd farm in Beijing. The type strain (strain 36) has been deposited in China General Microbiological Culture Collection Center (CGMCC) (Beijing) as deposition AS 1.2282^T.

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