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Molecular Identification of Predominant *Bifidobacterium* Strains Isolated from Korean Feces

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Abstract In order to isolate and identify *Bifidobacterium* spp. that originated in Korea, feces were sampled from healthy Korean adults and children living in three villages, the first having a history of longevity and the other two where the diet did not include fermented milk or any pharmaceutical preparations. Through the use of Gram staining and microscopic examination for cell morphology, 23 bacterial strains presumed to be the *Bifidobacterium* genus were isolated from the feces of 13 out of a total of 59 Korean people. To identify the *Bifidobacterium* strains at the genus level, these bacteria were then analyzed by TLC and the fructose-6-phosphate phosphoketolase (F6PPK) test. The result showed that 22 of the isolated strains were confirmed to be members of the genus *Bifidobacterium*. All of these bifidobacteria were also identified as *Bifidobacterium* spp. by the fermentation test. Using a RFLP analysis, an attempt was made to identify the *Bifidobacterium* spp. that had been isolated from both Korean adults and children. In a genomic Southern blot analysis after digestion with two restriction enzymes (*Eco*RI, *Hin*dIII), all of the 14 randomly selected Korean isolates showed patterns identical to those of three different *B. longum* species. Another restriction enzyme, *Cfo*I (4-bp recognition enzyme), was then used to identify the strain. Interestingly, all the Korean isolates were identified as *B. longum* ATCC 15708, indicating that a RFLP analysis was effective for identifying *Bifidobacterium* spp. at both the strain and species levels.

Key words: *Bifidobacterium* strains, *Bifidobacterium longum*, Korean feces, RFLP (restriction fragment length polymorphism) analysis

Bifidobacterium spp. are known as Gram-positive anaerobes, fermentative rods, mostly Y-shaped or clubbed at the ends, and asporogenous bacteria. It is clear that *Bifidobacterium* spp. constitute one of the major organisms in the colonic flora of healthy children and adults [19]. It has previously been reported that *Bifidobacterium* spp. can actually inhibit the growth of putrefactive bacteria by decreasing the pH of the intestine, thereby improving the immune system [19]. The beneficial effects of *Bifidobacterium* intake include prevention of carcinogenesis, improvement of protein metabolism, reinforcement of immune functions, and prevention of intestinal infections in the host [10, 17, 24]. Many beneficial effect of bifidobacteria has been ascribed to their production of lactic and acetic acids, with a consequence of lowered intestinal pH. This inhibits the proliferation of putrefactive bacteria and reduces ammonia absorption by maintaining it in the NH_4^+ form [15, 24]. Recently, as interest in human health has become widespread, these bacteria have been industrially applied as supplements to dairy products [31].

The glucose metabolism in bifidobacteria, originally identified in the late 1960s, is quite unique. This pathway is often referred to as the bifidus pathway and it has been used by researchers as a marker for the genus *Bifidobacterium* [9]. The key enzyme of the bifidus pathway is the fructose-6-phosphoketolase enzyme, which has now been isolated from all bifidobacterial species and well characterized [20, 30]. According to the bifidus pathway, 1.5 mol of acetate and 1 mol of lactate are generated for each mole of glucose fermented [7]. Based on this physiological characterization, the identification of bifidobacteria at the genus level has been performed by the following analyses; a fructose-6-phosphate phosphoketolase (F6PPK) analysis, an analysis of acetic and lactic acids in cell extracts by gas chromatography (GC), and finally, thin layer chromatography (TLC) analyses [13]. Carbohydrate fermentation patterns still provide principal guidelines for routine bifidobacterial characterization and identification [18]. However, the above mentioned methods are time consuming at initial stages of isolation and identification of human and animal feces, fermented milk,

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or probiotics containing some *Bifidobacterium* species, in addition to the fact that they are also deficient in sensitivity and specificity [4].

Recently, molecular biological methods have been used for the phylogenetic study and identification of microorganisms [12]. These methods include G+C content value estimation, DNA-DNA hybridization, RFLP (restriction fragment length polymorphism), RAPD (random amplified polymeric DNA), and DNA pulsed-field gel electrophoresis [23]. Among these methods, RFLP analysis has been widely used for making a clear assessment of the genetic relationship among bacterial species. In this study, *Bifidobacterium* strains were identified and classified according to their DNA fingerprints based on the RFLP patterns of 16s rRNA genes. Furthermore, these RFLP patterns were used to identify the *Bifidobacterium* spp. that had been isolated from Korean adults and children.

Feces were sampled from healthy Korean adults and children residing in three different villages where there was a history of longevity, and in two villages where the diet did not include milk and pharmaceutical preparations (Table 1). One gram of individual stool samples was collected in a sterile disposable bag (Seward Co., U.K.) containing

Table 1. Isolation of putative *Bifidobacterium* spp. from Korean adult and child feces.

Location	People		Strain
	Sex	Age	
Duryong village	Male	39	HLC 11841 HLC 11858
Munsan 1	Male	Teenager	HLC 21301 HLC 21034
	Male	25	HLC 21132 HLC 21141 HLC 21151
	Female	28	HLC 2441
	Male	25	HLC 21341 HLC 21242 HLC 21352
Munsan 2	Female	16	HLC 21431 HLC 21442
Sunhung village	Female	10	HLC 3131 HLC 3132
	Female	11	HLC 3241
	Female	32	HLC 3342
	Female	42	HLC 3451 HLC 3452
	Male	12	HLC 3641 HLC 3651
	Female	16	HLC 3742
	Male	14	HLC 3842

100 ml of physiological saline. This fecal suspension was then agitated by a laboratory blender stomacher 400 (Seward Co., U.K.) and serially diluted in physiological saline. Portions (0.1 ml) of the decimal dilutions in the physiological saline were then plated on a TPY agar plate and incubated in an anaerobic system (Forma Scientific Inc., U.S.A.) filled with mixed gases consisting of N_2 (75%), H_2 (10%), and CO_2 (5%) for 72 h at 37°C. Appearing colonies were picked from the TPY agar plates, restreaked for pure culture, and maintained on TPY agar slants for further studies. These purified colonies were then Gram-stained and examined under a microscope for any presence of the predominant strains of *Bifidobacterium*. All presumed *Bifidobacterium* colonies were inoculated in 10 ml of TPY broth, and incubated at 37°C for 20 h. A tentative identification was made in which the isolated bacterial stains were identified as *Bifidobacterium* spp. on the basis of a thin layer chromatographic (TLC) determination of short chain fatty acid metabolites in the culture broth [13] and a Fructose-6-Phosphate Phosphoketolase (F6PPK) test [29]. Practical identification of the *Bifidobacterium* at the species level was performed through fermentation tests carried out according to Bergey's Manual of Systematic Bacteriology [27].

Twelve reference strains of *Bifidobacterium* spp. obtained from ATCC are listed in the legend of Figs. 1 and 2. *Bifidobacterium* spp. were cultured in MRS broth as previously described [5]. A simple and rapid method for genomic DNA isolation was used, as previously described [11]. DNA manipulations including restriction digestion and Southern blot hybridization were carried out as described [26]. A 16S rDNA probe was prepared by PCR amplification by using *B. breve* genomic DNA as a template. The amplification procedure was carried out in 100 µl reaction volume with thin-walled tubes by using *Taq* polymerase in a Thermal cycler (Biometra, Germany) as previously described [22]. Eubacterial and universal primers were used, fD1 (5'AGAGTTTGATCTGGCTCAG3') & rD1 (5'AAGGAGGTGATCCAGCC3') as described by Weisburg *et al.* [32]. The RFLP patterns for all the strains examined were analyzed either visually or by the unweighted pair group method with arithmetic means (UPGMA), along with a dendrogram produced by using the computer-based taxanomy program, NTSYS-pc [7, 28].

Isolation *Bifidobacterium* spp. from Human Feces

Twenty-three predominant bifidobacterial strains were isolated from the two donor groups and examined by both Gram staining and microscopy for cell morphology. The first group consisted of 30 donors living in the villages of Duryong and Sangsa in Changhung-gun, Chunranam-do, and Danhang near Namhae-bridge of Kyungsangnam-do. The residents of these villages have a reputation for having longevity in life. The second group consisted of 29 donors living in the villages of Munsan-ri in Gangwon-do and

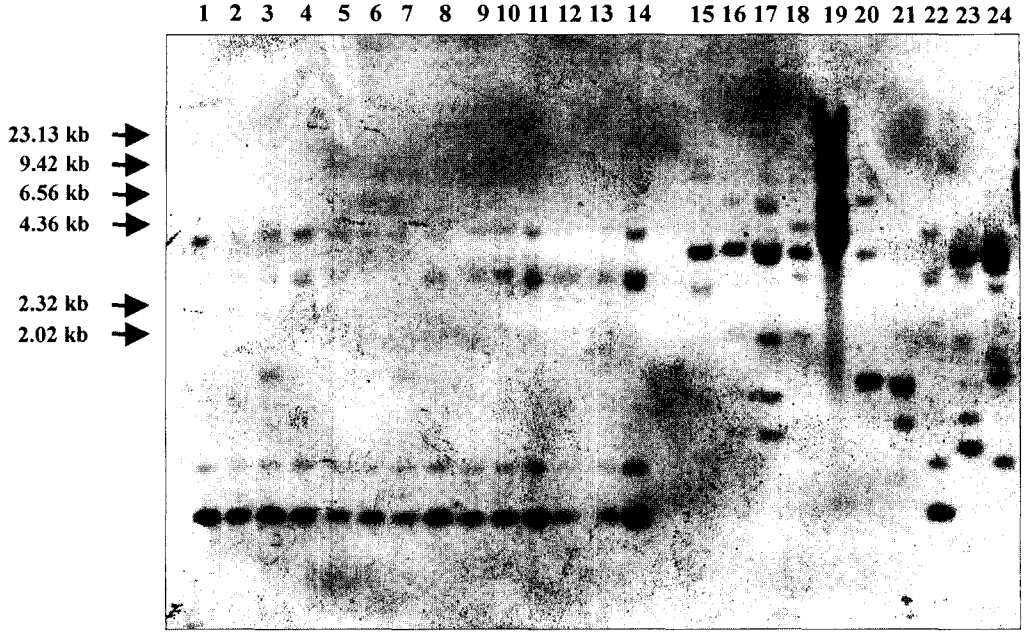

Fig. 1. Comparison of *Eco*RI RFLP patterns of 14 Korean isolates with those of 10 ATCC type strains.
Lanes 1 to 14, Korean isolates; Lane 15, *B. adolecentis* ATCC15703; Lane 16, *B. asteroids* ATCC15903; Lane 17, *B. bifidum* ATCC29521; Lane 18, *B. breve* ATCC15700; Lane 19, *B. globosum* ATCC25865; Lane 20, *B. indicum* ATCC25912; Lane 21, *B. infantis* ATCC25962; Lane 22, *B. longum* ATCC15708; Lane 23, *B. magnum* ATCC27540; Lane 24, *B. suis* ATCC27533.

Sunhung in the Sobak mountains of Kyungsangbuk-do where the diet did not contain such products as yogurt and probiotics. With an exception of only one person residing in the village of Duryong, where many people live to an advanced age, all the predominant bacterial strains presumed to belong to the *Bifidobacterium* genus were isolated from the feces of residents whose diet excluded yogurt and probiotics. When the culture supernatants of

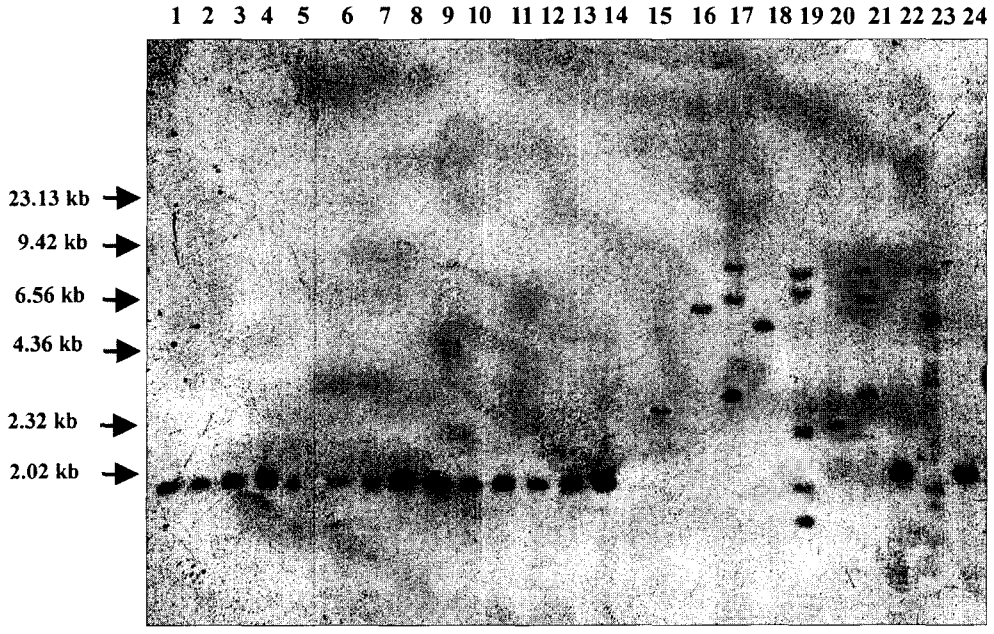

Fig. 2. Comparison of *Hin*dIII RFLP patterns of 14 Korean isolates with those of 10 ATCC type strains.
Lanes 1 to 14, Korean isolates; Lane 15, *B. adolecentis* ATCC15703; Lane 16, *B. asteroids* ATCC15903; Lane 17, *B. bifidum* ATCC29521; Lane 18, *B. breve* ATCC15700; Lane 19, *B. globosum* ATCC25865; Lane 20, *B. indicum* ATCC25912; Lane 21, *B. infantis* ATCC25962; Lane 22, *B. longum* ATCC15708; Lane 23, *B. magnum* ATCC27540; Lane 24, *B. suis* ATCC27533.

Table 2. Isolation pattern for a selection of *Bifidobacterium* strains.

Strains	Gram reaction	TLC analysis	F6PPK test
HLC 21242	+[a]	A[b], L[c]	+
HLC 21141	+	A, L	+
HLC 21442	+	A, L	+
HLC 3132	+	A, L	+
HLC 3641	+	A, L	+
HLC 3131	+	A, L	+
HLC 3241	+	A, L	+
HLC 21352	+	A, L	+
HLC 11858	+	A, L	+
HLC 21151	+	A, L	+
HLC 3452	+	A, L	+
HLC 21341	+	A, L	+
HLC 21031	+	A, L	+
HLC 3842	+	A, L	+
HLC 3651	+	A, L	+
HLC 3451	+	A, L	+
HLC 21132	+	A, L	+
HLC 3742	+	A, L	+
HLC 2441	+	L	–[d]
HLC 3342	+	A, L	+
HLC 21431	+	A, L	+
HLC 21034	+	A, L	+
HLC 11841	+	A, L	+

[a]Gram positive. [b]Acetic acid. [c]Lactic acid. [d]No F6PPK activity.

individual isolates were analyzed by TLC to determine organic acids produced [13], all the isolates, except for the strain that was referred to as HLC 2441, revealed two spots representing lactic acid and acetic acid. In contrast, HLC 2441 exhibited only one spot of lactic acid. These results indicate that 22 out of the 23 isolates were identified as bifidobacteria, whereas HLC 2441 was not. In addition, fructose-6-phosphokinase (F6PPK) test, a distinct key enzyme activity in bifidobacteria which can identify a strain with F6PPK, also produced results that were in line with the results determined by the TLC analysis (Table 2). Accordingly, the 22 isolates were identified as members of the genus *Bifidobacterium*. In addition, the sugar fermentation pattern confirmed that all the isolates were *Bifidobacterium* spp. except for one isolate, HLC 2441 (data not shown).

Identification of *Bifidobacterium* Strains by RFLP Analysis

The total genomic DNAs isolated from 10 *Bifidobacterium* type strains were digested with several restriction enzymes and the digest was hybridized with 16S rDNA. The resulting RFLP patterns were first analyzed visually. The size of the restriction fragments hybridized with 16S rDNA ranged from 500 bp to 23 kb. As shown in Fig. 1, the RFLP patterns following the *Eco*RI digestion revealed distinct patterns for each strain examined, except for two species, *B. asteroides* and *B. bifidum*, which exhibited identical patterns. The *Hin*dIII ribosomal patterns are illustrated in Fig. 2. The number of bands varied from one to five and their sizes ranged from 500 bp to 4.5 kb. As with the *Eco*RI digestion, identical patterns were also observed for *B. asteroides* and *B. bifidum*. A single band of 1 kb was detected in the genomic DNA digests of both *B. longum* and *B. suis*, suggesting a close relationship.

Based on the *Eco*RI and *Hin*dIII restriction patterns, the phylogenetic similarity was quantitatively estimated as a percentage of the common fragments found in each strain. Each band with two restriction endonucleases that differed from the others in its migration distance was numbered. A cluster analysis of the similarity coefficients for all pairs of strains was carried out by using the UPGMA (unweighted pair group method with arithmetic means). These numbers were then used as variables to construct a dendrogram by applying a computer-based taxonomy program (NTSYS-pc). The result of the computer analysis indicated that *B. longum* and *B. suis* had more than 60% similarity. On the basis of the above RFLP patterns, genetic identification experiments were then carried out on 14 randomly selected Korean isolates. It was surprising to find that all the Korean isolates exhibited an identical restriction pattern to that of *B. longum*, when the *Eco*RI RFLP patterns of the Korean isolates were compared with those of 10 type strains (Fig. 1). The *Hin*dIII RFLP patterns also indicated that the 14 Korean strains were identical to the *B. longum* species (Fig. 2). With the *Hin*dIII restriction enzyme, all 14 strains showed one band of 1.8 kb, as in the *B. longum* species. In order to identify the Korean isolates at the strain level, further RFLP analysis was conducted by using a 4-bp cutting restriction enzyme, *Cfo*I. As expected, three *B. longum* strains (ATCC 15707, ATCC 15708, and RW-001) showed distinct *Cfo*I ribosomal patterns, permitting the strain identification. As shown in Fig. 3, the randomly selected 7

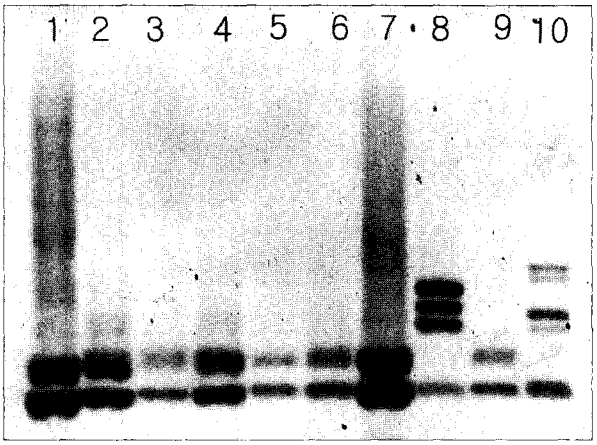

Fig. 3. *Cfo*I RFLP patterns of three different *B. longum* strains along with those of seven Korean isolates.
Lanes 1 to 7, Korean isolates; Lane 8, *B. longum* RW-001; Lane 9, *B. longum* ATCC 15708; *B. longum* ATCC 15707.

Korean isolates revealed RFLP patterns identical to those of *B. longum* ATCC 15708, which indicated that the predominant *Bifidobacterium* spp. present in Korean adults and children was *B. longum* ATCC 15708.

Various types of intestinal microorganisms exist in a fecal sample. The colonies that grow in plates with higher dilutions generally indicate the predominant intestinal microorganisms. Accordingly, in this study, the bifidobacterial colonies that grew in plates with higher dilutions were regarded as the predominant *Bifidobacterium* strains present in the gut. In the plates with higher dilutions where the colonies could be easily isolated, 20 putative *Bifidobacterium* strains were isolated from the feces of 12 Koreans residing in two locations where there was no dietary exposure to yogurt or probiotics. In contrast, only two *Bifidobacterium* strains were isolated from the feces of only one out of the 30 donors living in villages with a history of longevity. The age of this single donor was within the range of 10 to 42 years old. However, the ages of all the other donors from the longevity villages who did not exhibit any bifidobacteria ranged from 60 to 102 years old. Therefore, it is believed that this result is actually related to age. Bifidobacteria constitute numerically a major part of the fecal flora of healthy humans. The most common bifidobacteria isolated from infants belong to the species *B. bifidum*, *B. infantis*, *B. breve*, and *B. longum*. *B. adolescentis* and *B. longum* are found in large numbers in the intestines of children, adults, and senile men, although the occurrence of *B. adolescentis* is significantly higher in senile men than in the other age groups [17]. The detection rate and average number of *Bifidobacterium* in the feces of the elderly are both low [16]. Furthermore, the intestinal flora will change according to the age of the host. As a result, *E. coli* and *Enterococcus* are predominant in one week-old babies, and anaerobic microorganisms including bifidobacteria are predominant in infants. However the number of bifidobacteria in adults and the elderly decreases so that *Clostridium perfringens* and *C. paraputrificum* become predominant. This change is also accelerated by a decrease in the efficiency of digestion, intestinal movement, immunity, and so forth. Accordingly, the isolation of bifidobacteria in a highly diluted plate in this study was difficult for the elderly whose intestinal flora was dominated by *Clostridium* spp. rather than bifidobacteria. In the second group of donors, bifidobacteria were easily isolated from children and younger subjects. Based on this, it can be concluded that bifidobacteria are predominant in the intestinal flora of Korean children and young people, but not in the elderly.

Carbohydrate fermentation patterns provide the principal guidelines for routine bifidobacterial characterization and identification processes [18]. Except for HLC 2441, a 20-sugar fermentation ability test performed by the procedure described in Bergey's manual demonstrated that all the isolated strains belonged to the *Bifidobacterium* sp. However, this method has been shown to have low sensitivity and specificity [4], and it has generated many taxonomic uncertainties, particularly for *B. adolescentis* and *B. animalis*. Therefore, DNA-DNA hybridizations [29] along with subsequent electrophoretic protein patterns [4] were used for better assessment of the genetic relationships between the *Bifidobacterium* species.

Molecular approaches such as DNA-DNA hybridization and gene restriction patterns have contributed much to the clarification of bifidobacterial taxanomy [1, 14, 28, 33]. Likewise, computerized identification systems using commercial kits such as Oxi/Ferm (OF; Roche Diagnostics, Div. Hoffmann-La Roche, Inc., Nutley, N.J.), API 20E (AP; Analytab Products, Inc., Plainview, NY, U.S.A.), Minitek (MT, BBL Microbiology Systems, Cockeysville, Md.), and the Flow N/F system (NF, Flow Laboratories, Roslyn, NY, U.S.A.) have also been developed and marketed to identify various bacterial strains [2, 21]. Nevertheless, the use of the above mentioned methods for determining the taxonomy of the *Bifidobacterium* genus still remains problematic, particularly for laboratory use. Therefore, a molecular method based on RFLP of rRNA gene was proposed to resolve such problems.

In the present study, a comparative analysis of the *Eco*RI and *Hin*dIII ribosomal patterns from Korea fecal isolates and ten standard bifidobacteria strains was carried out. In each experiment, all 14 Korean isolates were identified as the *B. longum* species. Furthermore, the DNA of the *Bifidobacterium* strains originating from different human subjects displayed the identical *Cfo*I RFLP pattern, and the RFLP patterns of the Korean isolates (7 randomly chosen) were identical to that of *B. longum* ATCC 15708. Since stool populations reflect the most predominant population in the gut [14], the *B. longum* species could possibly be the predominant *Bifidobacterium* spp. in Korean gut. Until now, the *B. longum* ATCC 15708 strain has been isolated only from infant feces [3], therefore, it is noteworthy that *B. longum* belonging to the ATCC 15708 strain was isolated from Korean children and adults with high incidence.

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