Phenotypic and genotypic characterization of *Bifidobacterium* isolates from healthy adult Koreans

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Abstract

A total of twenty-two strict anaerobic and Gram-positive *Bifidobacteria*, identified as *B. adolescentis*, *B. pseudocatenulatum*, or *B. longum*, were isolated from healthy adult Koreans. We here investigated the cell morphology, antimicrobial resistance patterns to novel antibiotics and genotypic differentiation of *Bifidobacteria* assessing repetitive DNA element PCR (rep-PCR) fingerprinting using the BOXA1R primer at the species level. All *Bifidobacterium* spp., except *B. adolescentis* SPM1005 and *B. longum* SPM1205, formed round and convex colonies. All *B. adolescentis, B. pseudocatenulatum*, and *B. longum* were opaque white glossy in colony color, and short, long, and irregular rods in morphological shape. In addition, all *B. adolescentis*, *B. pseudocatenulatum*, and *B. longum* formed a variety of shapes ranging from rods to V-shaped, Y-shaped, clubbed rods, or irregular. All *Bifidobacterium* spp., except *B. adolescentis* SPM0214, were sensitive to daptomycin (DAP), linezolid (LIN), and tigecycline (TIG). *B. adolescentis* SPM0214 was resistant to DAP. Genomic fingerprinting patterns of *B. adolescentis, B. pseudocatenulatum*, and *B. longum* were diverse and different from those of the KCTC strain. The band size of *B. adolescentis, B. pseududocatenulatum*, and *B. longum* varied from 3.0 kb to 300 bp, 2.0 kb to 200 bp, and 2.0 kb to 500 bp, respectively. In conclusion, twenty-two strains of *B. adolescentis*, *B. pseudocatenulatum,* and *B. longum* isolated from healthy adult Koreans were very diverse in both phenotype and genotype. Moreover, this diversity of phenotype and genotype may support that health promoting effects of individual strain of *Bifidobacterium* spp. human isolates could be different and specific even within same species.

Keywords: *Bifidobacterium* species; healthy adult Koreans; phenotypic characterization; genotypic characterization; minimum inhibitory concentrations

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INTRODUCTION

Lactic acid bacteria (LAB) are nonpathogenic gram-positive inhabitants of normal human microflora. LAB are regarded as a probiotic which can inhibit harmful intestinal bacteria, improve lactose tolerance, synthesize vitamins, and reduce serum cholesterol levels (Choi *et al*., 2005; Rhee *et al*., 2002; Homma, 1998; Gopal *et al*., 1996; Mitsuoka, 1990; Modleret *et al*., 1990). LAB can play roles in improving immune function and helping cancer prevention in humans (Park *et al*., 1999; Rafter, 1999; Sekine *et al*., 1985). *Bifidobacterium* spp. is usually applied as the most common beneficial probiotic LAB in health promoting dairy products and food supplements because the organism can play an important role in maintaining the microbial balance of a healthy intestinal tract (You *et al*., 2004; Masco *et al*., 2003; Modler, 1994; Perdigon *et al*., 1986; Kato *et al*., 1981; Kohwi *et al*., 1978; Buchanon and Gibbons, 1976).

The assessment of antimicrobial susceptibility of *Bifidobacterium* spp. is an important prerequisite for its approval as a probiotic. Daptomycin (DAP) as a lipopeptide antibiotic shows bacteriocidal activity against Gram-positive bacteria for the treatment of complicated skin and skin structure infections caused by oxacillin-susceptible and oxacillin-resistant *Staphylococcus aureus* (Sader *et al*., 2005). Linezolid (LIN) as the first approved oxazolidinone shows bac-

teriocidal activity against vancomycin-resistant enterococci and methicillin-resistant staphylococci (Meka and Gold, 2004; Ross *et al*., 2004; Wesson *et al*., 2004). TIG as a novel parenteral glycylcycline for treating infections of the skin and skin structures and shows bacteriocidal activity against many Gram-positive, Gram-negative, atypical, and anaerobic organisms, including penicillin-resistant *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant enterococci (Betriu *et al*., 2005; Bouchillon *et al*., 2005; Fritsche *et al*., 2005 a,b; Sader *et al*., 2005; LaPlante and Rybak, 2004). DAP, LIN, and TIG can function against multidrug resistant pathogens and are regarded as novel antistaphylococcal antibiotics Generally, genomic DNA fingerprinting using repetitive element sequence-based polymerase chain reaction (rep-PCR) is a genotypic method for classifying and typing a wide range of bacteria using the primers of BOX, ERIC, REP, and TAP (Englund, 2003; Masco *et al*., 2003; Olive and Bean, 1999).

We previously isolated twenty-two strains of *Bifidobacterium* spp. from healthy adult Koreans and identified the strains as *B. adolescentis*, *B. pseudocatenulatum*, or *B. longum*. However, it is necessary to investigate several requirements associated with the identification of phenotypic characteristics including physiological patterns and antibiotic susceptibility and genotypic characteristics of the newly isolated *Bifidobacterium* spp. in order to apply the *Bifidobacterium* spp. as the potent probiotic.

Therefore, the objective of the current study was to investigate the cell morphology, antibiotic-resistance patterns to novel antibiotics (DAP, LIN, and TIG) and genotypic differentiation of *Bifidobacteria* isolated from healthy adult Koreans using BOXA1R-PCR fingerprinting at the species level.

MATERIALS AND METHODS

Isolation and identification of *Bifidobacterium* species: Fecal samples of 20 healthy Koreans (20-30 years old) were collected by BBL's anaerobic sample collection, transported under anaerobic conditions, and used within 24 hrs. Fecal samples were serially diluted 10-fold from 10^{-1} to 10^{-8}, and 100 µl was spread onto selective BL (Blood-Liver) agar (Nissui Pharm. Co. Ltd., Japan) containing 5% sheep blood, incubated 37°C for 48 h in a Bactron Anaerobic Chamber (Sheldon Manufacturing Inc., USA). Brown or reddish-brown colonies 2-3 mm in diameter were selected for further study (Scardovi, 1986). A fructose-6-phosphate phosphoketolase (F6PPK) test was performed (Ahn, 2005) to ensure that the colonies selected were *Bifidobacteria*. To identify the isolated *Bifidobacterium* spp. at the species level, 16S rRNA gene sequencing was performed by Bioleaders (Daejeon, Korea). The identification and origin of strain are shown in Table 1.

Cultural and microscopic observations: The isolated colonies confirmed by 16S rRNA gene sequencing were spread onto GAM (Nissui Pharm. Co. Ltd., Japan) plates followed by incubation either at 37°C in an aerobic incubator (Vision, Korea) or in a Bactron Anaerobic Chamber (Sheldon Manufacturing Inc., USA) for 48 h. After incubation, the shape and color of colonies onto the plates were visually observed. Gram staining reaction was determined according to the method described by Scardovi (1986). Morphology was observed by light microscopy (Nikon P-III, Japan).

Measurement of minimum inhibitory concentrations (MICs): The following three antimicrobial agents were provided by their manufacturers for use in

Table 1. Identification and origin of *Bifidobacterium* spp. isolated from healthy adult Koreans.

Number	Sex	Age	*Bifidobacterium* spp.
1	F	21	*B. adolescentis* SPM0212, SPM0212-b, SPM0212-c, SPM0214
2	F	22	*B. adolescentis* SPM0308
3	M	25	*B. adolescentis* SPM1005, SPM1005-a, SPM1005-b, SPM1005-c, SPM1005-d
4	M	24	*B. adolescentis* SPM1307-a, SPM1307-b, SPM1307-c
5	M	20	*B. adolescentis* SPM1601, SPM1604, SPM1605, SPM1606, SPM1608
6	F	22	*B. pseudocatenulatum* SPM1204, *B. longum* SPM1205, SPM1206, SPM1207

[1]SPM, Sahmyook Pharmacy Microbiology.

this study: daptomycin (DAP) (Cubist Pharmaceuticals, USA), linezolid (LIN) (Pharmacia, USA), and tigecycline (TIG) (Wyeth Pharmaceuticals, USA). MICs of three antibiotics were determined by the agar dilution method according to the guidelines established by the Clinical and Laboratory. Standards Institute (CLSI, 2003). MICs were defined as the lowest concentration of antimicrobial agent producing no visible growth of microorganism.

Extraction of genomic DNA and rep-PCR fingerprinitng: Twenty-two *Bifidobacterium* spp. were analyzed by repetitive DNA element PCR (rep-PCR) fingerprinting. All *Bifidobacterium* strains were grown overnight at 37°C on general anaerobic medium (GAM) (Nissui Pharm. Co. Ltd., Japan) under anaerobic conditions (90% N_2, 5% H_2, 5% CO_2). And the complete genomic DNA of all *Bifidobacterium* strains was isolated by using the Wizard genomic DNA purification kit (Promega, Co. Ltd., Madison, USA). The sequence of BOXA1R primer used for PCR was 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. PCR reactions were carried out in 30 µl reaction mixtures containing the DNA template, 10 mM Tris-HCl, 50 mM KCL, 1.5

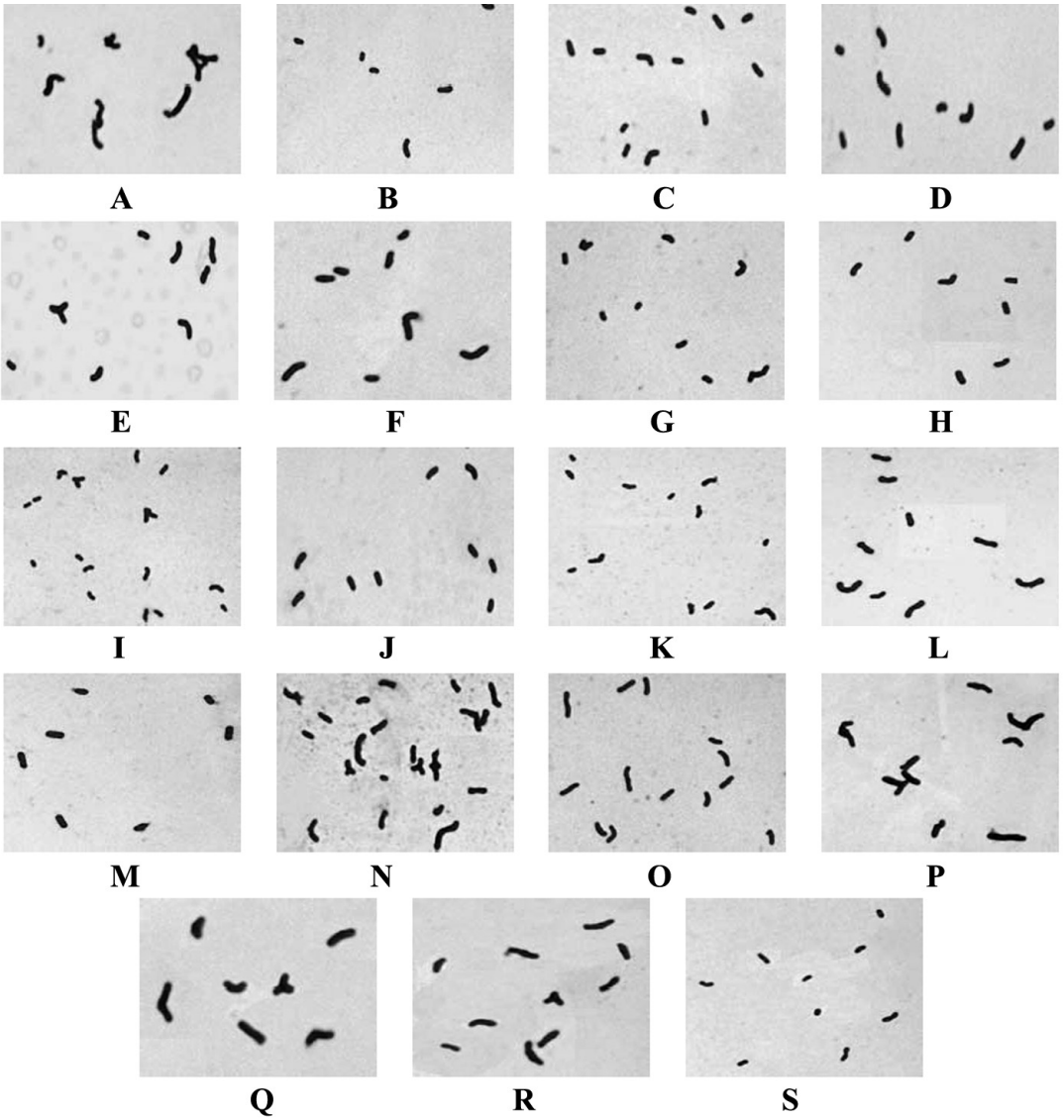

Figure 1. Light electron microscopy Gram-staining (magnification x 1,000) of *Bifidobacterium adolescentis* isolated from healthy adult Koreans on GAM. A: SPM0212 B: SPM0212-b C: SPM0212-c D: SPM0214 E: SPM0308 F: SPM1005 G: SPM1005-a H: SPM1005-b I: SPM1005-c J: SPM1005-d K: SPM1307-a L: SPM1307-b M: SPM1307-c N: SPM1601 O: SPM1604 P: SPM1605 Q: SPM1606 R: SPM1608 S: KCTC3325.

mM $MgCl_2$, 0.01% gelatin, 200 μM of each dNTP, primer, and 2.5 unit *Taq* DNA polymerase (Promega, Co. Ltd., Madison, USA). The reaction mixture was overlaid with a thin layer of sterile mineral oil to prevent evaporation. DNA amplification was performed in a programmable PTC-200 thermal cycler (MJ Research, USA) under the following cycling conditions. For BOXA1R: initial denaturation at 92°C for 2 min; followed by 35 cycles consisting of 30 s at 92°C, 1 min at 52°C, and 2 min at 72°C; and a final cycle of 72°C for 5 min. An additional step chilled the PCR products to 4°C. All amplified PCR products were resolved by electrophoresis on a 1.5% agarose gel in TAE buffer. PCR products were stained with ethidium bromide and visualized under UV light at 254 nm. Amplification reaction was performed twice to establish the reproducibility of this method.

RESULTS

Cultural physiology and microscopic morphology of *Bifidobacterium* isolates from healthy adult Koreans: All *Bifidobacterium* spp., except *B*. *adolescentis* SPM1005 and *B*. *longum* SPM1205, on GAM under an anaerobic condition form fairly round and convex colonies in shape (Table 2). *B*. *adolescentis* SPM1005 and *B*. *longum* SPM1205 on GAM under an anaerobic condition form irregular and spreading colonies in shape. All colonies of *B*. *adolescentis*, *B*. *pseudocatenultum*, and *B*. *longum* are also opaque white glossy in color (Table 2). Morphological shape of *Bifidobacterium* spp. human isolates are also shown in Table 2. All *B*. *adolescentis* (Fig. 1), *B*. *pseudocatenulatum* (Fig. 2), and *B*. *longum* (Fig. 3) are observed in short,

Table 2. Physiological and morphological characteristics of Bifidobacterium spp. isolated from healthy adult Koreans.

Strain	Cultural physiology		*Microscopic morphology*
	Colony shape	Colony color	Morphological shape
Bifidobacterium adolescentis			
SPM0212	Round, Convex	White, Glossy	Rod, V, Y, Irregular
SPM0212-b	Round, Convex	White, Glossy	Rod, V
SPM0212-c	Round, Convex	White, Glossy	Rod, V
SPM0214	Round, Convex	White, Glossy	Rod, V
SPM0308	Round, Convex	White, Glossy	Rod, V, Y
SPM1005	Irregular and spreading, Convex	White, Glossy	Rod, V
SPM1005-a	Round, Convex	White, Glossy	Rod, V
SPM1005-b	Round, Convex	White, Glossy	Rod, V
SPM1005-c	Round, Convex	White, Glossy	Rod, V, Y
SPM1005-d	Round, Convex	White, Glossy	Rod, V, Clubbed
SPM1307-a	Round, Convex	White, Glossy	Rod, V
SPM1307-b	Round, Convex	White, Glossy	Rod, V, Clubbed
SPM1307-c	Round, Convex	White, Glossy	Rod
SPM1601	Round, Convex	White, Glossy	Rod, V, Y, Clubbed
SPM1604	Round, Convex	White, Glossy	Rod, V
SPM1605	Round, Convex	White, Glossy	Rod, V, Irregular
SPM1606	Round, Convex	White, Glossy	Rod, V, Y
SPM1608	Round, Convex	White, Glossy	Rod, Y
KCTC3352	Round, Convex	White, Glossy	Rod, V
Bifidobacterium pseudocatenulatum			
SPM1204	Round, Convex	White, Glossy	Rod, Irregular
KCTC3223	Round, Convex	White, Glossy	Rod, V
Bifidobacterium longum			
SPM1205	Irregular and spreading, Convex	White, Glossy	Rod, V
SPM1206	Round, Convex	White, Glossy	Rod, V
SPM1207	Round, Convex	White, Glossy	Rod
KCTC3128	Round, Convex	White, Glossy	Rod

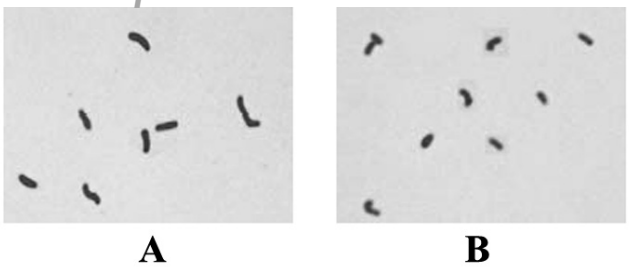

Figure 2. Light electron microscopy Gram-staining (magnification x 1,000) of *Bifidobacterium pseudocatenulatum* isolated from healthy adult Koreans on GAM. A: SPM1204 B: KCTC3323.

long, and irregular rods in morphological shape. In addition, all *B. adolescentis*, *B. pseudocatenulatum*, and *B. longum* form a variety of shapes ranging from rods to V-shaped, Y-shaped, clubbed rods, or irregular by light microscopy. No specific common colonies and morphological shapes of *Bifidobacterium* spp. human isolates are observed at the species levels.

Antibiotic-resistance patterns of *Bifidobacterium* isolates from healthy adult Koreans: To investigate the antibiotic-resistance patterns of *Bifidobacterium* spp. human isolates, minimum inhibitory concentrations (MICs) of novel antibiotics, DAP, LIN, and TIG on twenty-one strains of *Bifidobacterium* were tested (Table 3). No specific resistant patterns of *Bifidobacterium* spp. human isolates against new antibiotics were observed at the species levels. However, all *Bifidobacterium* spp., except *B. adolescentis* SPM0214, were found sensitive to DAP, LIN, and TIG. *B. adolescentis* SPM0214 was resistant to DAP.

Genomic fingerprinting patterns of *Bifidobacterium* isolates from healthy adult Koreans: Genomic fingerprinting patterns of *B. adolescentis* human isolates are shown in Figure 4. No specific common bands of *Bifidobacterium* spp. human isolates were observed at the species levels. Moreover, the genomic fingerprinting patterns of *B.*

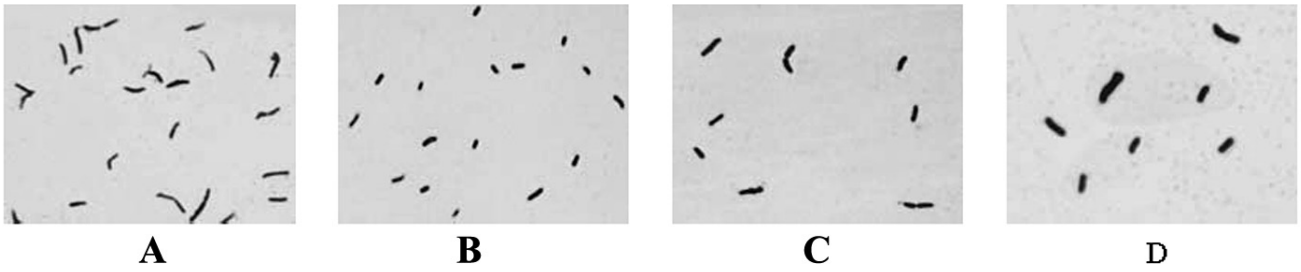

Figure 3. Light electron microscopy Gram-staining (magnification x 1,000) of *Bifidobacterium longum* isolated from healthy adult Koreans on GAM. A: SPM1205 B: SPM1206 C: SPM1207 D: KCTC3128.

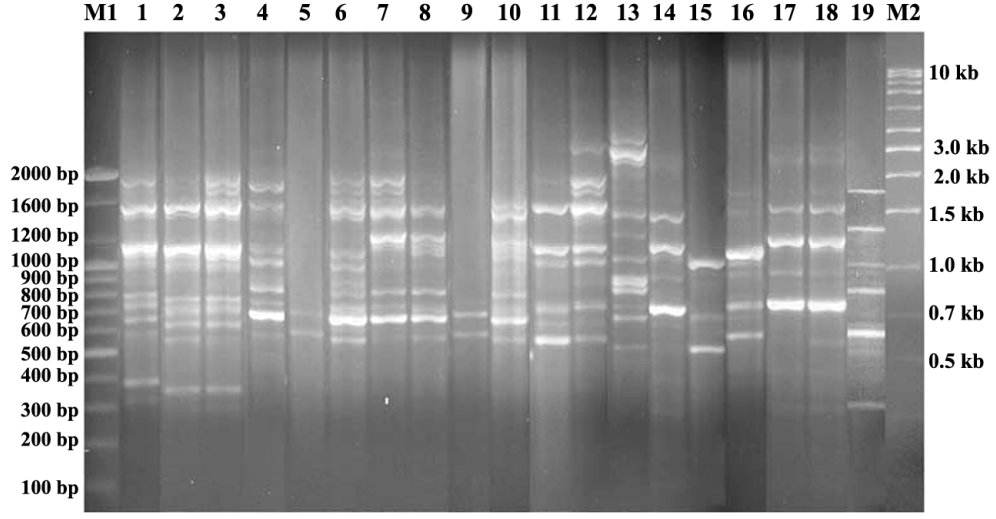

Figure 4. Genomic fingerprinting patterns of *Bifidobacterium adolescentis* isolated from healthy adult Koreans. Lanes: M1, 100 bp ladder size marker; 1, SPM0212; 2, SPM0212-b; 3, SPM0212-c; 4, SPM0214; 5, SPM0308; 6, SPM1005; 7, SPM1005-a; 8, SPM1005-b; 9, SPM1005-c; 10, SPM1005-d; 11, SPM1307-a; 12, SPM1307-b; 13, SPM1307-c; 14, SPM1601; 15, SPM1604; 16, SPM1605; 17, SPM1606; 18, SPM1608; 19, KCTC3352; M2, 1 kb ladder size marker.

Table 3. Minimum inhibitory concentrations (MICs) of antibiotics against *Bifidobacterium* spp. isolated from healthy adult Koreans.

Strain \ Antibiotics[a]	MICs (μg/ml)		
	DAP	LIN	TIG
Bifidobacterium adolescentis			
SPM0212	32	2	0.12
SPM0212-b	32	1	0.12
SPM0212-c	32	0.5	<0.06
SPM0214	64	1	0.25
SPM0308	32	1	0.25
SPM1005	32	1	0.5
SPM1005-a	32	1	0.25
SPM1005-b	16	1	0.25
SPM1005-c	16	0.5	0.25
SPM1005-d	16	2	0.5
SPM1307-a	16	1	0.25
SPM1307-b	16	0.5	<0.06
SPM1307-c	16	0.5	0.12
SPM1601	8	0.5	0.12
SPM1604	16	1	0.25
SPM1605	16	1	0.25
SPM1606	32	1	0.12
SPM1608	16	1	0.12
KCTC3352	4	0.5	0.25
Bifidobacterium pseudocatenulatum			
SPM1204	32	2	0.5
KCTC3223	32	2	0.25
Bifidobacterium longum			
SPM1205	32	2	0.25
SPM1207	16	1	<0.06
KCTC3128	128	0.5	0.25

[1]Abbreviations for antibiotics are: DAP, daptomycin; LIN, linezolid; TIG, tigecycline.

adolescentis human isolates were somewhat different although there are some common bands observed in Lane 1-4, Lane 6-7, or Lane 11-12 which were isolated from a same person (Table 1). That is, genomic fingerprinting patterns of *B*. *adolescentis* human isolates were diverse and different from those of the KCTC strain. The band size of *B*. *adolescentis* human isolates generated in rep-PCR varied from 3.0 kb to 300 bp. Genomic fingerprinting patterns of *B*. *pseudocatenulatum* human isolates differentiated from the strain of KCTC. The band size of *B*. *pseudocatenulatum* human isolates generated in rep-PCR varied from 2.0 kb to 200 bp. (Fig. 5). Genomic fingerprinting patterns of *B*. *longum* human isolates were diverse and different from those of the KCTC strain. The band size of *B*. *longum* human isolates generated in rep-PCR varied from 2.0 kb to 500 bp. (Fig. 6). Also, PCR-amplification using a BOXA1R primer produced one common band of 500 bp in *B*. *longum*.

DISCUSSION

In our previous study (results not published yet), a total of twenty-two strains of *Bifidobacterium* were isolated from healthy adult Koreans (20-30 years old)

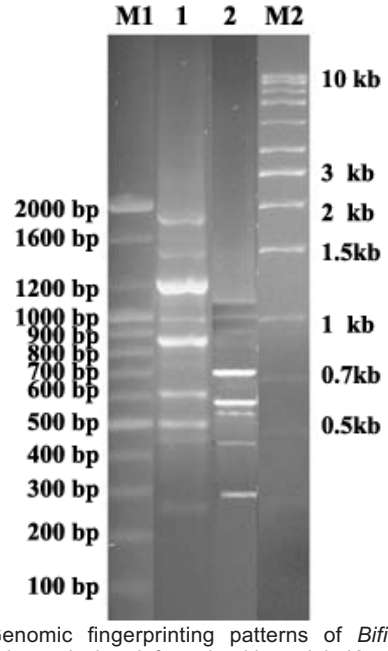

Figure 5. Genomic fingerprinting patterns of *Bifidobacterium pseudocatenulatum* isolated from healthy adult Koreans. Lanes: M1, 100 bp ladder size marker; 1, SPM1204; 2, KCTC3223; M2, 1 kb ladder size marker.

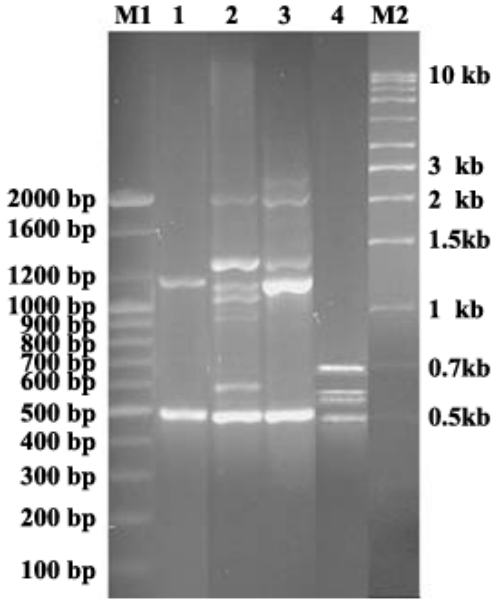

Figure 6. Genomic fingerprinting patterns of *Bifidobacterium longum* isolated from healthy adult Koreans. Lanes: M1, 100 bp ladder size marker; 1, SPM1205; 2, SPM1206; 3, SPM1207; 4, KCTC3128; M2, 1 kb ladder size marker.

and identified as *B. adolescentis*, *B. pseudocatenulatum*, or *B. longum* by F6PPK test and 16S rRNA gene sequencing. For the approval of our new isolation, *Bifidobacterium* as a potent probiotic, we assessed several important prerequisites which are the identification of phenotypic characteristics including physiological and antibiotic-resistance patterns and genotypic characteristics of the *Bifidobacterium* spp.

All *Bifidobacterium* spp. human isolates were Gram-positive and strict anaerobic. The strains of *Bifidobacterium* spp. human isolates were also diverse in colony and morphological shape at the species levels. DAP, LIN, and TIG can function against multidrug resistant pathogens. These three drugs are especially regarded as novel antistaphylococcal antibiotics and because *B. adolescentis* SPM0214 was resistant to DAP and sensitive to LIN, and TIG, the combination therapy of DAP and oral *B. adolescentis* SPM0214 could be beneficial for people who experience adverse effects due to overdose and prolonged periods of DAP therapy. Since DAP has been approved by the US Food and Drug Administration in 2003, this antibiotic is used for the treatment of complicated skin and skin structure infections caused by oxacillin-susceptible and oxacillin-resistant *S. aureus* (Sader *et al*., 2005).

We analyzed genomic fingerprinting patterns of twenty-two strains of *Bifidobacterium* human isolates with a BOXA1R primer using rep-PCR. The rep-PCR fingerprinting using the BOXA1R primer is an important genotypic method for the identification of a broad range of *Bifidobacterium* at the species level (Masco *et al*., 2003). The genomic fingerprinting patterns of *B. adolescentis*, *B. pseudocatenulatum*, and *B. longum* are various even within a same origin of isolation. The specific band of 500 bp in *B. longum* band could provide a basis for further studies to develop a sequence-characterized amplified region marker through DNA sequencing and database searching or for analyzing the phylogenic relationships of *B. longum*.

The results of current study showed that twenty-two strains of *B. adolescentis*, *B. pseudocatenulatum,* and *B. longum* isolated from healthy adult Koreans were very diverse in phenotype including cultural physiology, microscopic morphology, and resistance of new antibiotics and in genotype such as genomic fingerprinting patterns. Moreover, this diversity of phenotype and genotype may support the fact that health promoting effects of individual strain of *Bifidobacterium* spp. human isolates could be different and specific even within same species.

Acknowledgements

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