*J. Microbiol. Biotechnol.* (2001), **11**(1), 1–6

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY



# Characterization of Plasmids from *Bifidobacterium* sp.

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Received: February 9, 2000
Accepted: September 28, 2000

**Abstract** Ten strains of plasmid-harboring *Bifidobacterium* sp. were isolated from the feces of adults and children, and named as *Bifidobacterium* sp. GE1-GE8, ST, and SH5. These plasmids were categorized into three homologous groups (pKJ50-homologous, pKJ36-homologous, and non-homologous groups) according to Southern hybridization patterns using the formerly characterized bifidobacterial plasmids, pKJ50 and pKJ36, as probes. Nine strains harboring the plasmids were shown to accumulate single-stranded DNA as a replication intermediate, based on the S1 nuclease treatment and Southern hybridization. These results suggest that the strains replicate by a rolling circle mechanism. Minimal inhibitory concentrations (MIC) of the isolated bifidobacteria against several antibiotics were determined. Two strains, GE2 and GE3, showed relatively high MIC values against tetracycline (793.6 μg/ml) and erythromycin (153.6 μg/ml), respectively. The tetracycline resistance of GE2 disappeared when the resident plasmid of GE2 was cured by ethidium bromide. These results show that pKJ36-homologous and pKJ50-homologous plasmids are prevalent among various *Bifidobacterium* strains and some *Bifidobacterium* plasmids appear to code for antibiotic resistance.

**Key words:** Antibiotic resistance, *Bifidobacterium*, plasmid, ssDNA intermediate

Bifidobacteria were first discovered in 1899 by Tissier at the Pasteur Institute, Paris, France [24]. Bifidobacteria are Gram-positive strict anaerobes, produce acetate and lactate at the ratio of 3:2, and do not produce gas or spores. They are considered to play an important role in the proper balance of normal intestinal flora and exert many beneficial effects in the health of human beings [2]. Based on the beneficial properties of *Bifidobacterium*, the development of *Bifidobacterium* probiotic strains with improved characteristics through biotechnology is of crucial importance. Only a few studies have been published concerning the isolation and characterization of plasmids from bifidobacteria, because of the difficulties in their isolation and cultivation. Sgorbati *et al.* [20, 21, 22, 23] reported about 20% distribution of plasmids among the strains of *B. longum*, *B. globosum*, *B. indicum*, and *B. asteroids*, and Iwata *et al.* [7] isolated a plasmid from *B. breve*. Using these bifidobacterial plasmids, a few cloning vectors have been constructed. Matteuzzi *et al.* [12] characterized pMB1 (1.9 kb) from *B. longum*, and Bourget *et al.* [5] isolated and characterized pNB1 (5.6 kb) from *B. breve*. Missich *et al.* [13] constructed the pRM2, *E. coli-B. longum* shuttle vector with pMB1, and Matsumura *et al.* [10] constructed pBLES100 with pTB1 (3.6 kb) from *B. longum*. Park *et al.* [15, 16, 17] isolated and characterized pKJ50 (5.0 kb) and pKJ36 (3.6 kb) from *B. longum* KJ and constructed the *Bifidobacterium-E. coli* shuttle vector with these two plasmids. They reported that the deduced amino acid sequence of the three putative genes of pKJ50 and pKJ36 did not show any homology with the previously reported sequences of other *Bifidobacterium* strains. However, they shared an amino acid sequence with various plasmids from phylogenetically distant microorganisms such as *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Campylobacter coli*, *Pediococcus halophilus*, and *Helicobacter pylori*. Therefore, it is necessary to further study the relatedness of the various bifidobacterial plasmids to understand their distribution and elucidate their functions. No function has yet been ascribed to the bifidobacterial plasmids, except for the replication and transfer-related genes deduced from the DNA sequencing data. In this report, the distribution patterns of various bifidobacterial plasmids were studied and grouped, based on agarose gel electrophoresis and a Southern blot analysis.

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## MATERIALS AND METHODS

### Bacterial Strains and Media

Bifidobacteria were cultured anaerobically at 37°C in MRS broth (Difco Co., U.S.A.) supplemented with 0.05% (final concentration) cysteine · HCl. *Bifidobacterium* sp. GE1-GE8, SH5, and ST which harbor plasmids were isolated from feces of adults and children, as described below. Plasmids pKJ50 and pKJ36 from *B. longum* KJ [15] were used as probes.

### Isolation and Cultivation of *Bifidobacterium* Strains

*Bifidobacterium* was isolated from feces of adults and infants by the method of Ji *et al.* [8] using the TP medium as the selective medium. Plates were incubated anaerobically for 2-3 days at 37°C, then the colonies were further isolated, and a fructose-6-phosphate phosphoketolase (F6PPK) test was performed [19] to ensure that the colonies selected were *Bifidobacterium*. The anaerobic cultivation of *Bifidobacterium* was carried out using an 8-ml screw cap tube (Corning Co., U.S.A.) and anaerobic jar and gas pack system (Difco Co., U.S.A.). A BHI and MRS media containing 0.05% (w/v) L-cysteine · HCl was used as the basal medium for the cultivation and preservation of the isolated *Bifidobacterium* strains.

### Preparation and Purification of Plasmids from *Bifidobacterium* sp.

*Bifidobacterium* sp. was incubated, prepared, and purified by the method of Park *et al.* [15].

### Viable Cell Counting and Spectrophotometric Determination of the Amount of Plasmid DNA

For counting viable cells, bifidobacteria were plated on MRS agar (Difco Co., U.S.A.), incubated at 37°C for 24 h in an anaerobic condition, and viable cells were counted. To quantitate the amount of DNA, absorbance was taken at wavelengths of 260 nm and 280 nm. An optical density (OD) reading of 1 at 260 nm corresponds to 50 μg/ml of double-stranded DNA. The ratio of $OD_{260}/OD_{280}$ provides an estimate of the purity of the nucleic acid (1.8-2.0). Based on this, plasmid DNAs were tested to confirm their purity. For determination of the relative plasmid DNA copy number, bifidobacteria viable cell counts were normalized.

### Southern Blot Analysis

Southern blotting was performed with a DIG DNA Labeling and Detection Kit (Boehringer Mannheim Co., Germany) according to the manufacturer's protocol.

### Plasmid Curing

*Bifidobacterium* sp. GE2 and *Bifidobacterium* sp. GE3 were subcultured three times in the presence of ethidium bromide (15 mg/ml) and spread on an agar plate. After the colonies appeared, they were tooth-picked on an agar plate with and without antibiotics for each strain. Subsequently, only the colonies that developed on the agar plate without antibiotics were examined whether they included a plasmid or not.

### Antibiotic Susceptibility of *Bifidobacterium* Strains (Minimal Inhibitory Concentration Test)

An antibiotic resistance test was performed. All the antibiotics were purchased from Sigma Chemical Co., U.S.A. The stock solutions (1,000 ×) of antibiotics were prepared by dissolving chloramphenicol in ethanol (31.2 mg/ml), kanamycin in distilled water (1,600 mg/ml), ampicillin sodium salt in distilled water (1.48 mg/ml), penicillin G in methanol (0.52 mg/ml), metronidazole in water (400 mg/ml), tetracycline in methanol (396.8 mg/ml), amoxicillin in methanol (6.4 mg/ml), and erythromycin in ethanol (78.6 mg/ml). The above stock solutions were filtered through a membrane (0.22 μm in pore size), serially diluted, added to a liquid-phase BHI agar in 20-ml screw-capped tubes to a proper concentration, poured into a petri dish, and finally hardened. The *Bifidobacterium* strains were activated by cultivation in BHI medium for 12 h and then replica-plated on plates containing antibiotics using a microplate replicator. The MIC value was determined at the minimal antibiotic concentration at which no bacterial colony developed.

### Detection of the Single-Stranded DNA (ssDNA) Replication Intermediate

To detect a replication intermediate, a Southern blot analysis was performed according to the method developed by Leenhouts *et al.* [9]. The plasmid DNA was prepared from the bifidobacteria during the mid-log phase ($OD_{600}$=0.6), as described above, and divided into two portions. One of those was resuspended in S1 endonuclease buffer and the other as a control was not treated. For the specific digestion of ssDNA, the S1 endonuclease (100 U/ml, final concentration, Promega Co., U.S.A.) was incubated with the ssDNA suspension for 15 min at 25°C. After electrophoresis of S1 nuclease treated and not-treated DNAs in an agarose slab gel, the DNAs were transferred to a nitrocellulose filter. The presence of ssDNA was determined by Southern hybridization with pKJ50 and pKJ36 DNA as the probes.

## RESULTS AND DISCUSSION

### Isolation of Plasmid-Harboring *Bifidobacterium* sp.

In order to isolate and identify plasmid-harboring *Bifidobacterium* sp., 193 *Bifidobacterium* isolates were isolated from feces of adults and children, and these strains were tested for the presence of plasmid DNA. Ten strains exhibited plasmid DNA bands (Fig. 1a), when the plasmid DNA was prepared and electrophoresed according

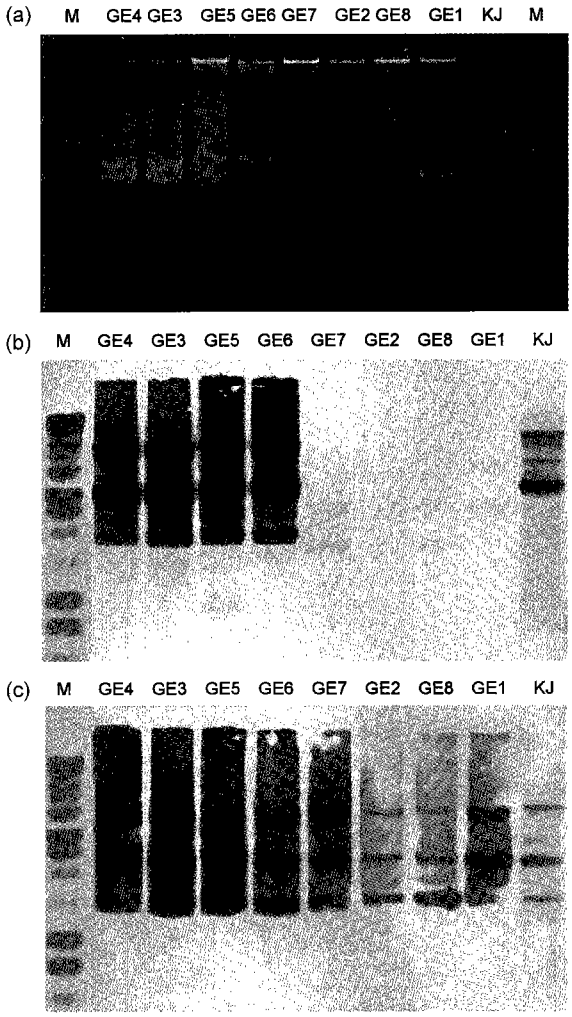


**Fig. 1.** Agarose gel electrophoretogram of bifidobacterial plasmids obtained from various *bifidobacterium* strains isolated from human feces (1a), Southern blot analysis of bifidobacterial plasmids using DIG-labeled pKJ50 (1b) and pKJ36 (1c) as hybridization probes.
M: 1 kb ladder. GE: Various strains of *Bifidobacterium* sp.
KJ: *B. longum* KJ.

to Materials and Methods. All the plasmid-harboring *Bifidobacterium* strains showed typical morphological characteristics of bifidobacteria (data not shown) and showed a positive reaction to the F6PPK test and Gram staining. Sgorbati *et al.* [20] reported that, among about 1,200 strains of *Bifidobacterium* sp. screened for the presence of plasmid DNA, about a 61% distribution of plasmid DNA was shown especially in the case of *B. longum*. However, the current study showed only a 5% distribution of plasmid DNA among all the *Bifidobacterium* strains tested. pGE1, the plasmid of *Bifidobacterium* sp. GE1, showed a relatively high plasmid DNA concentration in agarose gel electrophoresis when an equal viable cell number of bifidobacteria was used (data not shown). This suggests that pGE1 may have a high copy number.

**Table 1.** Minimal inhibitory concentration of *Bifidobacterium* sp.

| | (μg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Cm | Km | Amp | PenG | Met | Tet | Amx | Em |
| GE1 | ≤2.0 | 800 | ≤0.09 | ≤0.03 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| GE2 | ≤2.1 | ≤100 | ≤0.09 | ≤0.03 | ≤25 | 153.6 | 3.2 | 0.08 |
| GE3 | ≤2.2 | 800 | ≤0.09 | 0.26 | ≤25 | ≤1.55 | 0.8 | 793.6 |
| GE4 | 1.56 | 800 | ≤0.09 | 0.07 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| GE5 | ≤2.0 | ≤100 | ≤0.09 | ≤0.03 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| GE6 | ≤2.1 | 400 | ≤0.09 | 0.26 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| GE7 | ≤2.2 | ≤100 | ≤0.09 | ≤0.03 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| GE8 | ≤2.3 | ≤100 | 0.19 | ≤0.03 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| Adol | ≤2.4 | 800 | ≤0.09 | ≤0.03 | 50 | ≤1.55 | ≤0.4 | ≤0.08 |
| Anim | ≤2.5 | ≤100 | ≤0.09 | ≤0.03 | 400≤ | ≤1.55 | ≤0.4 | ≤0.08 |
| Brev | ≤2.6 | ≤100 | ≤0.09 | ≤0.03 | 400≤ | ≤1.55 | ≤0.4 | ≤0.08 |
| Long | ≤2.7 | ≤100 | ≤0.09 | ≤0.03 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| KJ | ≤2.8 | ≤100 | ≤0.09 | ≤0.03 | ≤25 | ≤1.55 | ≤0.4 | ≤0.08 |
| GE2P- | N | N | N | N | N | ≤1.55 | N | N |

Cm: Chloramphenicol; Km: Kanamycin; Amp: Ampicillin; PenG: Penicillin G; Met: Metronidazole; Tet: Tetracycline; Amx: Amoxicillin; Em: Erythromycin; GE: *Bifidobacterium* sp. GE; KJ: *Bifidobacterium longum* KJ; Adol: *B. adolescentis*; Anim: *B. animalis*; Brev: *B. breve*; Long: *B. longum*; GE2P-: *Bifidobacterium* sp. GE2 cured by ethidium bromide.
N: not tested.
≤: below that value.

### Minimal Inhibitory Concentration (MIC) Test of Plasmid-Harboring *Bifidobacterium* sp.

Until now, the correlation between the function of bifidobacterial plasmids and their physiological characteristics has not yet been reported. Therefore, the functions of the small plasmids of bifidobacteria are cryptic. It is reported that most of the *Bifidobacterium* strains are susceptible to penicillin, erythromycin, clindamycin, and vancomycin [11], whereas they are not susceptible to metronidazole, a synthetic 5-nitroamidazole that is active against virtually all obligate anaerobes. This accounted for the absence of a ferredoxin-like system in bifidobacteria [2]. Among the ten plasmid-harboring bifidobacteria, *Bifidobacterium* sp. GE2 and GE3 showed relatively high MIC values (Table 1) against tetracycline (153.6 μg/ml) and erythromycin (793.6 μg/ml), respectively. The strong erythromycin resistance of GE3 is of interest, since most bifidobacteria are highly susceptible to this antibiotic. If this antibiotic resistance is not transferable to other intestinal bacteria, these strains could be co-administered when tetracycline or erythromycin treatments are used against intestinal pathogenic bacteria. Furthermore, these antibiotic resistance genes may be good candidates as selection markers in the vector construction.

### Curing of Plasmids from *Bifidobacterium* sp.

Only a plasmid was removed from *Bifidobacterium* sp. GE2 and the cured strain was designated as *Bifidobacterium* sp. GE2P- (Fig. 2). The MIC to tetracycline of *Bifidobacterium* sp. GE2P- was below 1.55 μg/ml, which was the average value of the other tetracycline-susceptible *Bifidobacterium*

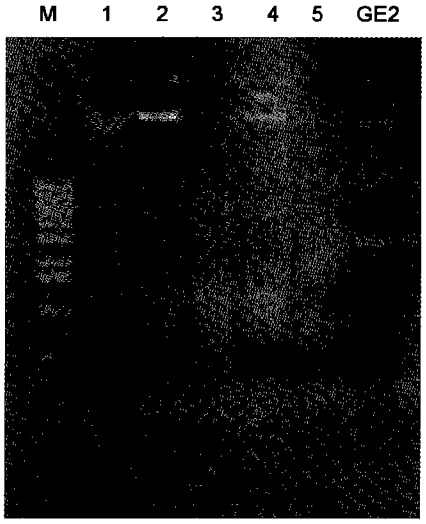


**Fig. 2.** Agarose gel electrophoretogram of wild-type and cured GE2.
M: 1 kb ladder. Cured strains. 1: CE-1; 2: CA-1; 3: CA-2; 4: CA-3; 5: CA-4 (CE: cured with ethidium bromide; CA: cured with acridine orange). GE2: *Bifidobacterium* sp. GE2.

strains (Table 1) and was much lower than the non-cured *Bifidobacterium* sp. GE2. This result suggests that the plasmid in *Bifidobacterium* sp. GE2 may confer a resistance against tetracycline to its host. The transformation of the plasmid into a tetracycline-susceptible *Bifidobacterium* will prove more clearly if the antibiotic resistance is coded in the plasmid. In other lactic acid bacteria, there have been a number of attempts to identify the plasmid function using plasmid curing and most have been studied in relation to antibiotic resistance [1, 25]. The transformation of the plasmids from GE2 and GE3 into another *Bifidobacterium* by the electroporation method was attempted but the MIC of those was not changed. This negative result may be because the host range of these plasmids is very narrow or there is an antibiotic resistance gene either in the chromosomal DNA or in an unidentified large molecular weight plasmid DNA.

## Structural Relationships Between Plasmids from *Bifidobacterium* sp.

A Southern blot analysis was performed to investigate the structural relationships among the bifidobacterial plasmids, using two plasmids, pKJ50 and pKJ36, from *B. longum* KJ as probes. All plasmids in the ten tested isolates were categorized into three homologous groups; pKJ36 homologous group, pKJ50 homologous group, and non-homologous group (Figs. 1b and 1c). The plasmid-harboring *Bifidobacterium* strains were grouped into three: strains harboring only the pKJ36 homologous plasmid; and GE1, GE2, GE7, and GE8 strains harboring both pKJ36 and pKJ50 homologous plasmids inside the same host; and GE3-GE6, ST, and one other strain, SH5, harboring a

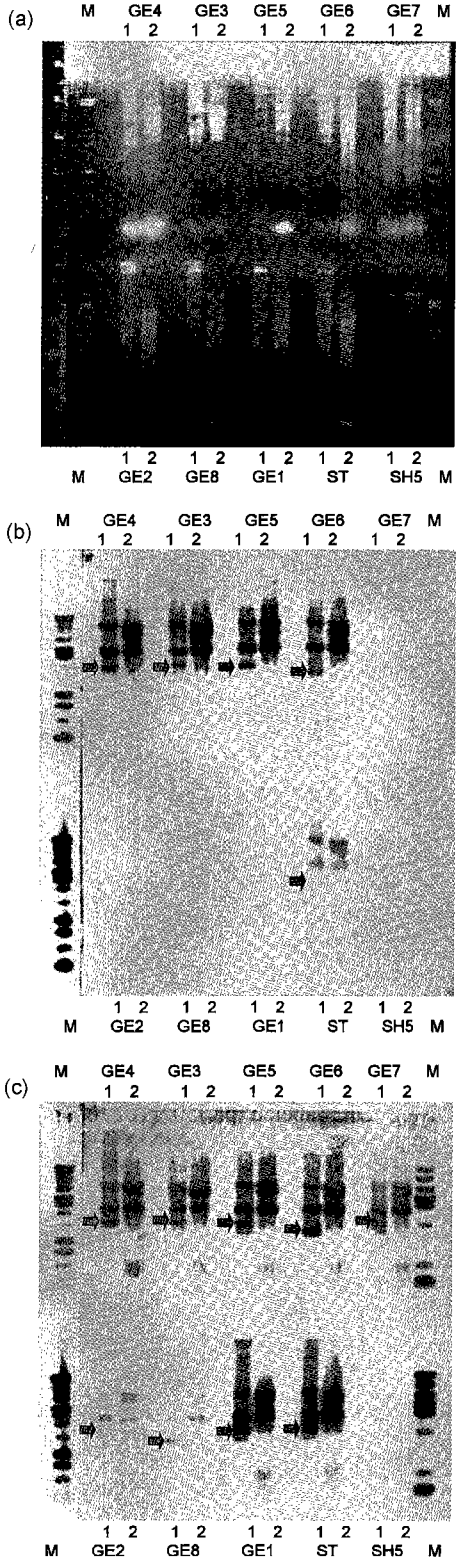


**Fig. 3.** Agarose gel electrophoresis of bifidobacterial plasmids before and after treatment with S1 nuclease (3a), and Southern blot analyses using pKJ50 (3b) and pKJ36 (3c) as hybridization probes.
M: 1 kb ladder; GE, ST and SH5: *Bifidobacterium* sp.; 1: before treatment with S1 nuclease; 2: after treatment with S1 nuclease.

non-homologous plasmid with pKJ36 and pKJ50. Although all plasmids were catagorized into three homologous groups, the restriction enzyme digestion patterns among the homologous plasmids were different (Figs. 3b and 3c). Sgorbati *et al.* [20, 22, 23] reported that there are seven different plasmid profiles in *B. longum* and thirteen in *B. asteroides*, *B. indicum*, and *B. globosum*, whereas only three homologous groups were identified in this experiment. No direct comparison has yet been made between the plasmids reported by Sgorbati *et al.* and the presently reported plasmids, except for the report that pKJ50 and pKJ36 do not share a homology with Sgorbati's pMB1 plasmid. None of the bifidobacteria harbored the pKJ50 homologous plasmid only, which may indicate that some replication factors expressed from pKJ36 are necessary for the proper maintenance of pKJ50 homologous plasmids in their host.

**Analysis of Single-Stranded Plasmid DNA in *Bifidobacterium* sp.**

Only a few indirect studies on the replication mechanism of bifidobacterial plasmids have been performed by O'Riordan *et al.* [14]. However, they suggested that bifidobacterial plasmids are replicated by a rolling circle replication exhibiting a nicking site and three motifs related to the initiation proteins for the rolling circle type replication. To know if those plasmids generate single-stranded DNA (ssDNA) intermediates or not, detection of ssDNA was performed using endonuclease S1 as described in Materials and Methods. A comparison of the Southern blot analysis of the S1-treated and not-treated plasmids, using pKJ50 and pKJ36 as probes, revealed that the ssDNA intermediate disappeared after treatment with endonuclease S1 thus suggesting that pKJ50, pKJ36 and all the other plasmids, except SH5, produced ssDNA intermediates (Figs. 3a, 3b, and 3c). These ssDNA intermediate forms might have been involved in the replication of the *Bifidobacterium* plasmids, since numerous plasmids of Gram-positive bacteria replicate via single-stranded intermediates, probably by rolling circle replication, similar to the ssDNA phages of *E. coli* [6].

## Conclusions

To investigate the distribution and characterization of the plasmids in the genus *Bifidobacterium*, bifidobacteria from feces of adults and children were screened. Ten strains harboring plasmid DNA were identified, designated as *Bifidobacterium* sp. GE1-GE8, SH5, and ST, and were confirmed as *Bifidobacterium* sp., based on the fructose-6-phosphate phosphoketolase (F6PPK) test. These isolated plasmids were then categorized into three homologous groups according to Southern blot analysis, using the formerly characterized bifidobacterial plasmids, pKJ50 and pKJ36, as probes. To differentiate the plasmids, five digested plasmid band patterns with *Sau*3AI were demonstrated using the same methods (data not shown). To identify the replication mode of the bifidobacterial plasmids, S1 endonuclease that removes ssDNA was used in the bifidobacterial plasmids preparation. All the plasmids were shown to accumulate ssDNA as a replication intermediate, evidenced by a Southern blot analysis using pKJ50 and pKJ36 as probes. These ssDNA replication intermediates suggest replication by a rolling circle replication mechanism. However, further experimental data are needed to confirm this theory. Two strains, GE2 and GE3, showed relatively high minimal inhibitory concentration (MIC) values against tetracycline and erythromycin, respectively. When GE2 was cured, the tetracycline resistance disappeared and the MIC decreased to the same value as other tetracycline-sensitive plasmid DNA-harboring strains. We are in a process to search for the location of the antibiotic resistance genes. These antibiotic resistance genes may be useful as selectable markers for vector construction from *Bifidobacterium* sp. In addition, the role and function of the plasmids other than antibiotic resistance should be studied in order to fully exploit the probiotic effect of *Bifidobacterium* in the promotion of human health.

## Acknowledgments

The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program year of 1998.

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