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Molecular Characterization of Plasmid from *Bifidobacterium longum*

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Abstract The complete nucleotide sequence of a plasmid, pMG1, isolated from *Bifidobacterium longum* MG1 has been determined. This plasmid, composed of 3,862 base pairs with 65.1% of G+C content, harbors two major open reading frames (ORF) encoding putative proteins of 29 kDa (ORF I) and 71 kDa (ORF II). ORF I showed relatively high amino acid sequence homology with replication proteins of other plasmids from Gram-positive and -negative bacteria. Upstream of ORF I, four sets of tandem repeat sequences resembling the iteron structure of related plasmids were found. S1 endonuclease treatment and Southern blot analysis revealed that pMG1 accumulates single-stranded DNA (ssDNA) intermediate, which indicates the rolling circle replication (RCR) mechanism of this plasmid. Homology search indicated that ORF II encodes plasmid mobilization protein, and the presence of highly conserved *oriT* sequence in the upstream of this gene supported this assumption. RT-PCR showed that only ORF I is expressed *in vivo*. Based on these results, pMG1 was exploited to construct a shuttle vector, pBES2. It was successfully transformed into *Bifidobacterium* and maintained stably.

Key words: *Bifidobacterium longum* MG1, plasmid, sequence analysis, shuttle vector, electroporation

Ever since Tissier first discovered the bifidobacteria in the feces of infants in 1899, many researchers have reported the beneficial effects of these bacteria on human health. Among lactic acid bacteria, bifidobacteria are the only major components of human large intestine and confer more beneficial effects on the host than other lactic acid bacteria. Because of the general belief that bifidobacteria are helpful to maintain a proper balance in the human intestinal flora, these organisms have been the subject of numerous studies designed either to elucidate the properties of the 'bifidus factors' in human milk or find a substitute for it [9]. Recently, several reports have elucidated the reduced symptoms of irritable bowel disease [3], treatment of allergy [11], shortening of rotavirus diarrhea [23], and improved oral vaccination [14]. Because the genus *Bifidobacterium* is generally recognized as safe (GRAS) and colonizes the large intestine, it is regarded as a promising candidate for a safe expression and delivery system for human use. For these reasons, the isolation and characterization of plasmids from bifidobacteria [2, 13, 15, 21, 27] and vector construction using those plasmids [1, 16, 18, 20, 22] have been pursued. For example, Nakamura *et al.* [17] tried to apply a bifidobacteria vector system, expressing the cytosine deaminase gene, to enzyme/pro-drug therapy of solid tumors. Because the full genome sequence of *B. longum* has been determined [26] and those of two more bifidobacteria are being sequenced [12], genetic tools working in these microorganisms are needed to more fully utilize the genome information. In this report, a plasmid, pMG1, has been sequenced from *B. longum* MG1 and a shuttle vector, pBES2, has been constructed which can transform *Bifidobacterium*.

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Materials and Methods

Bacterial Strains, Plasmids, and Culture Media

B. longum MG1, which was the original source of plasmid pMG1, was grown anaerobically at 37°C in MRS broth (Hardy Diagnostics, CA, U.S.A.) supplemented with 0.05% of L-cysteine HCl. The anaerobic condition has been established by using ANOXOMAT WS8000 system (MART Microbiology BV, Lichtenvoorde, The Netherlands). As a recipient strain for transformation, *E. coli* DH5α was used and cultivated in Luria-Bertani liquid medium (Hardy Diagnostics, CA, U.S.A.). All the bacterial strains used in this study were stored in 15% (v/v) glycerol stock at -70°C and subcultured periodically.

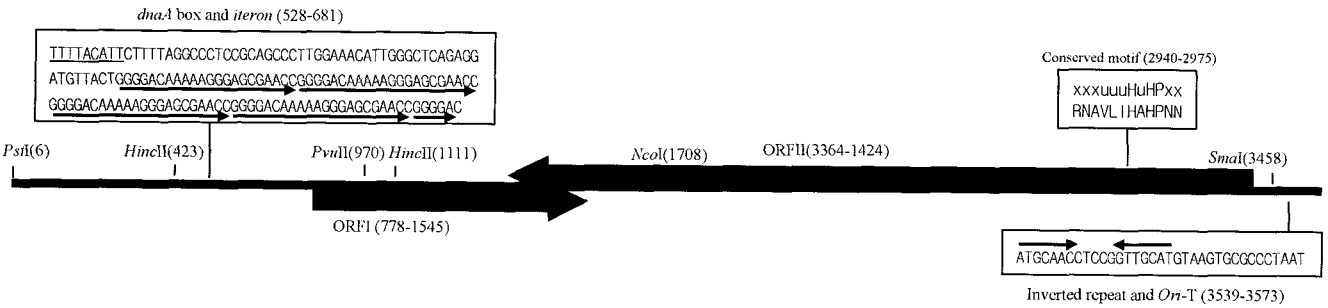

Fig. 1. Schematic presentation of nucleotide sequence of pMG1. Each ORF is indicated as filled arrows.
The nucleotide sequence of putative *dnaA* box and iteron region (528-681) and *oriT* region (3539-3573) are shown. The amino acid sequence of highly conserved motif (2940-2975) in ORF II is indicated as a box (u-hydrophobic residue, x-nonconsensus residue). The nucleotide sequence is available in the NCBI and GenBank database under accession number of AY210701.

General Cloning Techniques and Sequence Analysis

The small and large scale plasmid DNA preparation, restriction enzyme digestion, ligation, and transformation of *E. coli* were carried out according to the procedure of Sambrook *et al.* [24]. All the enzymes for the molecular work were purchased from Promega (Madison, WI, U.S.A.) and the chemicals were from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.) if not indicated otherwise. Plasmid DNA was prepared from *Bifidobacterium* according to the method of Park *et al.* [21]. In order to analyze the sequence of pMG1, it was digested with *Pvu*II and cloned into the *Hinc*II site of pUC19 to consturct pUMG1. Sets of nested deletion mutants from pUMG1 were obtained by using Deletion Kit for Kilo-Sequencing (Takara Co., Tokyo, Japan). The plasmid DNA template for sequencing was prepared by using Wizard® *Plus* Minipreps DNA Purification System (Promega, Madison, WI, U.S.A.). The sequencing reaction was carried out using ABI PRISM™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit and the sequence was read by ABI PRISM 377 (Applied Biosystems, CA, U.S.A.). Total RNA from *B. longum* MG1 was isolated using the High Pure RNA Isolation Kit (Boehringer Mannheim, Germany). RT-PCR was performed using Selective PCR Kit (AMV)Ver.1.1 (Takara, Tokyo, Japan) and primers orf I-f (5'-ATGTCCAATGAGATCGTGA-3') and orf I-r (5'-TCAGTGGTCGTCAACACC-3') for ORF I, and orf II-f (5'-ATGTCGTGGTCGTAGATG-3') and orf II-r (5'-AAG-ACCAGGGCAGCGA-3') for ORF II, respectively.

Shuttle Vector Construction and Transformation of *Bifidobacterium*

The shuttle vector between *E. coli* and *Bifidobacterium* was constructed by cloning pMG1 into the *Pst*I site of pEK104 [20] and named as pBES2 (Fig. 5). It was purified from *E. coli* and electrotransformed into *Bifidobacterium* sp. according to the modified method of Argnani *et al.* [1]. In order to enhance the transformation efficiency, oxyrase (Oxyrase Inc., Ohio, U.S.A.) was added to the incubation buffer after an electric pulse was given to the competent cell according to the manufacturer's recommendation and the transformed cells were selected on the MRS agar containing 3 μg/ml of chloramphenicol (Sigma Chem. Co., St. Louis, U.S.A.).

Detection of Single-Stranded Intermediate

The ssDNA was detected by the method of Park *et al.* [19], which relies on comparing plasmid DNA before and after a selective digestion of ssDNA by S1 endonuclease.

Results and Discussion

Cloning and Sequence Analysis of pMG1

B. longum MG1 was isolated from an infant and proved to harbor one kind of plasmid, named pMG1 (Fig. 6, Lane 1). It showed a relatively higher copy number than other bifidobacterial plasmids, when agarose gel electrophoresis was done (data not shown), and was estimated as a

Table 1. Amino acid sequence homologies and similarities of ORF I with various replication proteins.

Homologous proteins	Identities (%)	Positives (%)	References
RepA of pKJ50 from *B. longum*	58	72	GenBank U76614
RepB of pKJ36 from *B. longum*	81	85	GenBank AF139129
RepA of *Lactobacillus acidophilus*	37	55	GenBank D55703
Replication protein of *Enterococcus faecalis*	35	61	GenBank D85392
RepA protein of *Pediococcus halophilus*	34	56	GenBank X75607
Replication protein of *Staphylococcus epidermidis*	33	53	GenBank AF045241
Replication protein of *Streptococcus bovis*	31	53	GenBank AB021464

Table 2. Amino acid sequence homologies and similarities of ORF II with various plasmid mobilization proteins.

Homologous proteins	Identities (%)	Positives (%)	References
MobA of pKJ50 from *B. longum*	44	58	GenBank U76614
MobB of pKJ36 from *B. longum*	92	95	GenBank AF139129
Nicking enzyme (traA) of *Lactococcus lactis*	33	48	GenBank AE001272
Mobilization protein of Streptococcal plasmid	29	50	GenBank L39769
MobL protein of *Thiobacillus ferrooxidans*	30	43	GenBank X52699
Mobilization proteinA of *E. coli*	28	39	GenBank M28829
OriT nickase Nes of *Staphylococus aureus*	26	44	GenBank AF051917

promising candidate as an expression vector for bifidobacteria. First, pMG1 was digested with *Pvu*II and ligated into the *Hinc*II site of pUC19 to construct pUMG1, and the restriction map (Fig. 1) was obtained and fully sequenced as described in Materials and Methods. It was composed of 3,682 base pairs and showed a G+C content of 65.1%, which is slightly higher than that of the genome of *B. longum* (60% of G+C contents) [26]. A database search using the BLAST program revealed two major ORFs that were designated as ORF I and ORF II according to amino acid sequence homology (Fig. 1). Putative ribosome binding sites (RBS), AGGA sequence, were present about 10–15 base pairs apart from the start codon of each ORF. ORF I, encoding 255 amino acids corresponding to a 29-kDa putative protein having a pI value of 10.3, showed more than 55% amino acid sequence homologies with other replication proteins of Gram-positive and -negative bacteria (Table 1). In the upstream of ORF I, an iteron structure was found which had 22 base-pair units tandemly repeated four and a half times (Fig. 1). Iteron structures act as binding sites for the replication protein, and may control plasmid copy number and incompatibility [7]. Bifidobacterial plasmids pKJ50 and pKJ36 also have these iteron structures, but the repeating sequences are variable among them [19, 20]. Similar results have also been found in the pCI305/pWV02 family [6] of *Lactococcus*. Furthermore, *dna*A box (Fig. 1) could be detected in the upstream of the iteron sequence as defined as a common feature of iteron-containing plasmids by Schaefer and Messer [25]. ORF II, encoding a putative 71 kDa protein with pI value of 11.8, showed more than 40% amino acid sequence homologies with other mobilization protein of several bacteria (Table 2). Multiple alignment of these mobilization proteins using the CLUSTAL [10] program revealed a highly conserved motif (Fig. 1). This motif is identical to that previously reported by Tatyana and Eugene [28] as the most prominent among the three consensus sequences of the mobilization proteins of the pUB110 family, the pMV158 family, and the phage family. Another interesting observation was the presence of a putative *oriT* site in pMG1 with an identical 12 bp sequence (Fig. 2), which was found in pKJ50, pKJ36, Gram-negative plasmids RSF1010, pTF1, pSC101, and R1162, as well as streptococcal plasmid pIP501 [29], staphylococcal plasmid pGO1 [4], and *A. tumefaciens* plasmid pTiC58 [5]. The inverted repeat sequence commonly found in the upstream region of *oriT* sequence was also present in pMG1. These results suggest the possibility of gene transfer mechanisms

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pMG1        AATGCAACCTCCGGTTGCATGTAAGTGCGCCCTAATC
pKJ50     TGAATGTTACCACCGGTAACATGTAAGTGCGCCCTCAAT
pKJ36       AATGCAACCTCCGGTTGCATGTAAGTGCGCCCTAATC
pIP501  ATACGAAGTAACGAAGTTACTGCGTATAAGTGCGCCCTTAGT
RSF1010   CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCCC
pSC101      TGAACGAAGTGAAGAAAGTCTAAGTGCGCCCTGAT
pTF1      GGGTAATCTCGAAGAGATTACTCTAAGTGCGCCCTTGC
pGO1        CACGCGAACGGAACGTTCGCATAAGTGCGCCCTTAC
R1162     CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCC
pTiC58    CAAGGCGTCGCGTCAGCGACGTATAATTGCGCCCTTG
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Fig. 2. Multiple alignment of *oriT* regions of various plasmids.
The nucleotide sequence of the putative *oriT* site of pMG1 is aligned with similar sequences found in various plasmids such as pIP501, RSF1010, pTF1, pSC101, pGO1, R1162, and pTiC58. The *oriT* region is found in the upstream of each *mob* gene. Inverted repeat sequences are marked by underlines. The asterisks and bold characters indicate the conserved sequences among these ten plasmids.

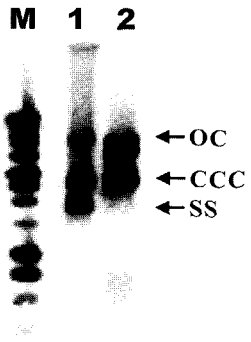

Fig. 3. Detection of single-stranded DNA intermediates of pMG1.
Whole cell DNA was prepared from *B. longum* MG1 (Lane 1) and treated with S1 endonuclease (Lane 2). Southern blot analysis was performed using pMG1 as probe. OC, open circular DNA; CCC, covalently closed circular DNA; SS, single-stranded DNA.

via bacterial conjugation in the genus *Bifidobacterium*. However, no evidence to support this has yet been reported.

Detection of ssDNA Intermediate of pMG1

A Southern blot analysis after treatment of S1 endonuclease using pMG1 as a probe revealed that pMG1 accumulated ssDNA as a replication intermediate (Fig. 3). Numerous plasmids of Gram-positive bacteria have been reported to replicate *via* ssDNA intermediates, probably by rolling circle replication similar to the ssDNA phages of *E. coli* [8]. The replication mechanism of pMG1 may also be similar to pKJ50 and pKJ36, which also showed ssDNA intermediates in a previous study [19].

In vivo Expression of ORF I and ORF II

RT-PCR was performed as described in Materials and Methods to estimate the *in vivo* expression of each ORF deduced from sequence analysis. Two sets of specific primers for ORF I and ORF II were used to amplify each ORF from templates of total RNA and pMG1 isolated from *B. longum* MG1. As a result, ORF I showed expected PCR and RT-PCR products (750 bp, Lanes 1 and 2 of Fig. 4), whereas ORF II showed only the PCR product (600 bp, Lane 3 of Fig. 4). These results suggest that ORF I is expressed constitutively, but the expression of ORF II may be regulated and expressed only when plasmid transfer occurs.

Shuttle Vector Construction and Transformation of *Bifidobacterium*

Based on the sequence analysis of pMG1, an *E. coli-Bifidobacterium* shuttle vector, named as pBES2, was constructed by cloning whole pMG1 plasmid into the *Pst*I site of pEK104 as described in Materials and Methods and Fig. 5. The transformation into *B. longum* MG1 by electroporation was carried out with an efficiency of 7.3×10^1 CFU/μg DNA. The GenPulser (Bio-Rad, U.S.A.) was optimized by setting at 2,500 V, 200 Ω, and 40 μF. The oxyrase treatment further improved the electroporation efficiency by 100-fold (7.3×10^3 CFU/μg DNA). The transformed MG1 showed plasmid DNA band patterns different from that of the non-transformed one: That is, the original pMG1 band became weak or disappeared, which might have been due to incompatibility between pMG1 and pBES2 (Fig. 6). The restriction band patterns of pBES2 isolated from *E. coli* and *Bifidobacterium* transformants showed the same results (data not shown). Furthermore, more than 90% of pBES2

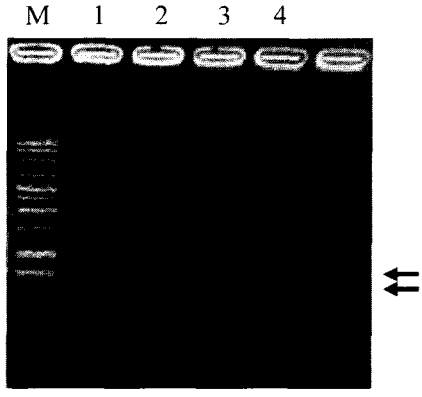

Fig. 4. Confirmation of expression of each ORF of pMG1 by PCR and RT-PCR.
Lanes: 1, PCR product with pMG1 plasmid template and primers orf I-f and orf I-r; 2, RT-PCR product with total RNA from *B. longum* MG1 and primers orf I-f and orf I-r; 3, PCR product with pMG1 plasmid template and primers orf II-f and orf II-r; 4, RT-PCR product with total RNA from *B. longum* MG1 and primers orf II-f and orf II-r; M, 1 kb DNA ladder.

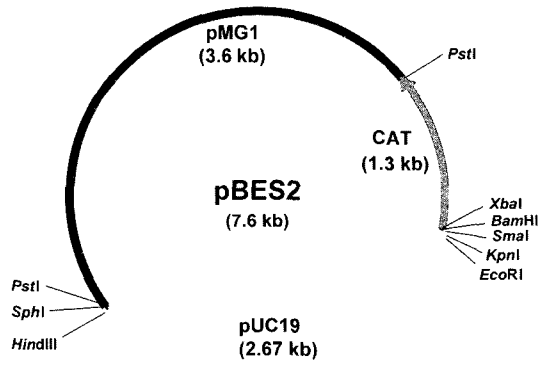

Fig. 5. Restriction enzyme digestion map of *E. coli-Bifidobacterium* shuttle vector pBES2.
pBES2 has been constructed by cloning pMG1 into *Pst*I site of pEK104 [18] which is composed of chloramphenicol resistance gene and pUC19 and each component is annotated in the map. Restriction enzyme sites which can serve as a cloning site for foreign genes are indicated.

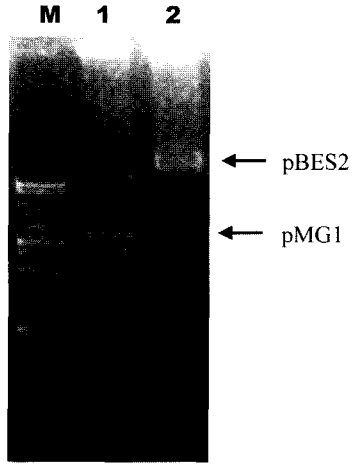

Fig. 6. Agarose gel electrophoresis of pMG1 and pBES2 prepared from *B. longum* MG1.
Lanes: 1, plasmid was isolated from overnight culture of *B. longum* MG1 in MRS broth; 2, plasmid was prepared from overnight culture of *B. longum* MG1 transformed with pBES2 in MRS broth containing chloramphenicol (3 μg/ml); M, 1 kb DNA ladder. Each DNA band for pMG1 and pBES2 is indicated.

was maintained in the transformed cells after 30 generations without an antibiotic selection. These results mean a good structural and segregational stability of pBES2, which is an essential requisite for a practical vector. Aslo, it contains many useful restriction enzyme sites (Fig. 5), which can provide versatile sites for foreign gene cloning and expression in the genus *Bifidobacterium*. The sequence analysis further revealed that pMG1 contains replication protein and mobilization protein, which are homologous with those of various Gram-positive and -negative bacteria. The *in vivo* expressions of those expected ORFs were investigated by RT-PCR and the replication mode was estimated by S1 endonuclease treatment and Southern hybridization. In conclusion, a new shuttle vector, pBES2, which can transform *E. coli* and *B. longum* MG1, was constructed, and the vector could be valuable as a delivery vehicle of functionally beneficial gene products into gastrointestinal tracts of the host.

Acknowledgments

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