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# Characterization of Plasmid pKJ36 from *Bifidobacterium longum* and Construction of an *E. coli-Bifidobacterium* Shuttle Vector

**PARK, MYEONG-SOO[1], DONG-WOO SHIN[2], KE-HO LEE[2], AND GEUN-EOG JI[3]***

[1]*Research Center for Agriculture and Life Sciences, Seoul National University, Suwon 441-744, Korea*
[2]*Department of Food Science and Technology, Seoul National University, Suwon 441-744, Korea*
[3]*Department of Food Science and Nutrition, Seoul National University, Seoul 151-742, Korea*

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**Abstract** The full sequence of the plasmid pKJ36, which was derived from *Bifidobacterium longum* KJ, was determined and analyzed to construct shuttle vectors between *E. coli* and *Bifidobacterium.* The plasmid pKJ36 was composed of 3,625 base pairs with a 65.1% G+C content. The structural organization of pKJ36 was highly similar to that of pKJ50, and the three major ORFs on pKJ36 showed high amino acid sequence homologies with those of pKJ50. The putative proteins coded by these three ORFs were designated as RepB (32.0 kDa, pI=9.25), MembB (29.0 kDa, pI=12.25), and MobB (39.0 kDa, pI=10.66), respectively. The amino acid sequence of RepB showed a 57% identity and 70% similarity with that of the RepA protein of pKJ50. Upstream of the *repB* gene, the so-called iteron sequence was directly repeated four-and-a-half times and a conserved *dnaA* box was identified. An amino acid sequence comparison between the MobB and MobA of pKJ50 revealed a 48% identity and 61% similarity. A conserved *oriT* sequence with an inverted repeat identical to that of pKJ50 was also found upstream of the *mobB* gene. A hydropathy analysis of MembB revealed four possible transmembrane regions. The expressions of the *repB* and *memb*B genes were confirmed by RT-PCR. The *in vitro* translation reaction of pKJ36 showed protein bands with anticipated sizes with respect to each putative gene product. S1 endonuclease treatment and Southern hybridization suggested that pKJ36 replicates by a rolling circle mechanism via a single-stranded DNA (ssDNA) intermediate. A shuttle vector between *E. coli* and *Bifidobacterium* sp. was constructed using the pKJ36, pBR322, and staphylococcal chloramphenicol acetyl transferase (CAT) gene. The successful transformation of the *Bifidobacterium* strains was shown by Southern hybridization and PCR. The transformation efficiency differed from strain to strain and, depending on the electroporation conditions, with a range between $1.2 \times 10^1 - 2.6 \times 10^2$ cfu/μg DNA.

*Corresponding author
Phone: 82-2-880-8749; Fax: 82-2-884-0305;
E-mail: Geji@mbio.snu.ac.kr

**Key words:** Plasmid, sequence, *Bifidobacterium longum*, gene expression, shuttle vector, replication

Bifidobacteria are well known for their important role in the proper balance of normal intestinal flora and their beneficial effects on the health of human beings. This may be due to an altered intestinal pH, specifically, through the release of acetic and lactic acids. Lee *et al.* [17] reported that *Bifidobacterium* contribute to the reinforcement of host immune functions and improve resistance to cancer. Therefore, the development of *Bifidobacterium* probiotic strains with improved characteristics through biotechnology is becoming popular. In order to develop a cloning vector for *Bifidobacterium* modification, a comprehensive understanding of the replication mechanism and characterization of a *Bifidobacterium* plasmid is necessary. Recently, several studies have been published concerning the isolation and characterization of plasmids from bifidobacteria [4, 19, 23, 27] and vector construction using a *Bifidobacterium* plasmid [2, 20]. Rossi *et al.* [24] reported on the sequence of the 1,847 bp plasmid from *Bifidobacterium longum* B2577, whose ORF showed an amino acid sequence homology with the peptides from pXZ10142 of *Corynebacterium glutamicum* and pAL5000 of *Mycobacterium fortuitum.* Another bifidobacterial plasmid pKJ50, which is one of the two plasmids existing in *B. longum* KJ, was also fully sequenced and analyzed to construct a shuttle vector between *Escherichia coli* and *Bifidobacterium* [22]. The analysis of the gene structure revealed putative Rep and Mob proteins that were highly homologous with the proteins of various other gram-positive and gram-negative bacteria. However, the Rep protein of pKJ50 showed little homology with that of pMB1 from *B. longum* B2577. The bifidobacterial plasmids so far isolated from 11 strains can be categorized into three homologous groups according to their Southern hybridization patterns (pKJ50 -

homologous, pKJ36 - homologous, and nonhomologous groups; paper in preparation). The strains with both pKJ50 and pKJ36-homologous plasmids have shown relatively higher plasmid stability than the strains with only pKJ36 homologous plasmids (data not shown). These results seem to suggest that the cooperation between the Rep proteins of pKJ50 and pKJ36 contributes to the proper maintenance of each plasmid. In order to understand the genetic relationship between the two plasmids, the molecular structure of pKJ36 was investigated and compared with that of pKJ50. Furthermore, a shuttle vector based on pKJ36 was constructed and transformed into *Bifidobacterium.*

## MATERIALS AND METHODS

### Bacterial Strains, Media, and Plasmids

*E. coli* DH5α was used for the transformation and cultured at 37°C in a Luria broth with vigorous shaking. Ampicillin was used at a concentration of 50 μg/ml. The bifidobacteria were cultured at 37°C in an MRS broth (Difco) supplemented with 0.05% (final concentration) cysteine-HCl. The multicopy plasmid vector pUC19 was used for the cloning of pKJ36.

### General Cloning Techniques and Sequence Analysis

The small and large-scale plasmid DNA preparation from *E. coli*, restriction enzyme digestion, and ligation and transformation of *E. coli* were all carried out according to the procedure of Sambrook *et al.* [25]. The plasmid DNA from the *Bifidobacterium* strains was prepared as described by Park *et al.* [23]. The DNA was recovered from agarose gels using a Gene Clean Kit (Bio 101 Inc. U.S.A.). For the sequencing reaction, plasmid DNA was prepared from *E. coli* DH5α using a Wizard™ Minipreps DNA Purification System (Promega) according to the manufacturer's recommendation. Unidirectional deletion mutants were constructed with a Kilo-Sequencing Deletion Kit (TAKARA Shuzo Co. Japan). The sequencing reactions were performed using the Cy5™ AutoRead™ Sequencing Kit (Pharmacia) and ALF DNA Sequencer (Pharmacia). The DNA and amino acid sequence data analyses were performed using the DNASIS and PROSIS programs (HITACHI Software Engineering Co. Japan), respectively. DNA and amino acid sequence homology searches were conducted online using the World Wide Web server for BLAST searches maintained at the National Center for Biotechnology Information (Bethesda, MD; URL=http://www.ncbi.nlm.nih.gov/Recipon/blast_search.html) along with/in conjunction with the programs BLASTN, BLASTP, BLASTX, and TBLASTN [1]. The multiple sequence alignment of related amino acid sequences was performed using the CLUSTAL V program [14]. A hydropathy plot analysis of each deduced amino acid was completed using the Internet program (http://www.microbiology.adelaide. edu.au/learn/index.htm).

### Southern Blot Analysis and Detection of Single-Stranded DNA

The Southern blot analysis was performed using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Germany) according to the manufacturer's protocol. The ssDNA was detected by the method of Leenhouts *et al.* [18], which relies on comparing plasmid DNA before and after selective digestion of ssDNA with S1 endonuclease.

### Coupled *in vitro* Transcription/Translation and RT-PCR

The *in vitro* translation was performed using the *E. coli* T7 S30 Extract System for Circular DNA (Promega) according to the manufacturer's protocol and as described in Park *et al.* [22]. The isolation of total RNA from *Bifidobacterium* and RT-PCR was conducted according to the method of Park *et al.* [22] using pKJ36 as template and three sets of primers (DW001: 5'-AGTTCTCGTTCGAGGAGCTG-3', DW002: 5'-CGTCAAACGCGCAGGAAACG-3'; DW003: 5'-ATGGCGATCTACCATCTGTC-3', DW004: 5'-GGTTCCTCACGTCGGCCTTG-3'; MS015: 5'-GGGGAATGCCGTTTCCTGCG-3', MS016: 5'-GCCAAGGAGCCGAACTGGCG-3').

### Shuttle Vector Construction and Transformation of *Bifidobacterium*

For the construction of the shuttle vector, a 1.3 kb staphylococcal chloramphenicol acetyl transferase (CAT) gene (*Eco*RI-*Hin*dIII fragment of pEK104, 22) was first ligated with a 1.8 kb pBR322/*Pvu*II-*Hin*cII fragment and designated pBRCm (Fig. 11). The CAT gene then enabled the pBRCm carrying *E. coli* transformants to grow at 25 μg/ml. Next, *E. coli-B. animalis* shuttle vectors, named as pBKJ36F and pBKJ36R, were constructed by cloning the whole pKJ36 plasmid into the *Pst*I site of pBRCm as described in Fig. 11. Electrocompetent cells of *B. animalis* MB209 were prepared according to the method of Argnani *et al.* [2]. The EasyJect One pulse generator (EquiBio, Belgium) was set at 10 kV/cm, 200 Ω, and 40 μF.

## RESULTS AND DISCUSSION

### Sequence Analysis of pKJ36

For its structural analysis, pKJ36 was digested with *Pst*I and subcloned into the *Pst*I site of pUC19 in both a forward and reverse direction, and the resulting nested deletion mutants were fully sequenced, as described in Materials and Methods. The entire nucleotide sequence (3,625 bp) of pKJ36 was deposited in the Genbank (accession number AF139129). The G+C content of pKJ36 was

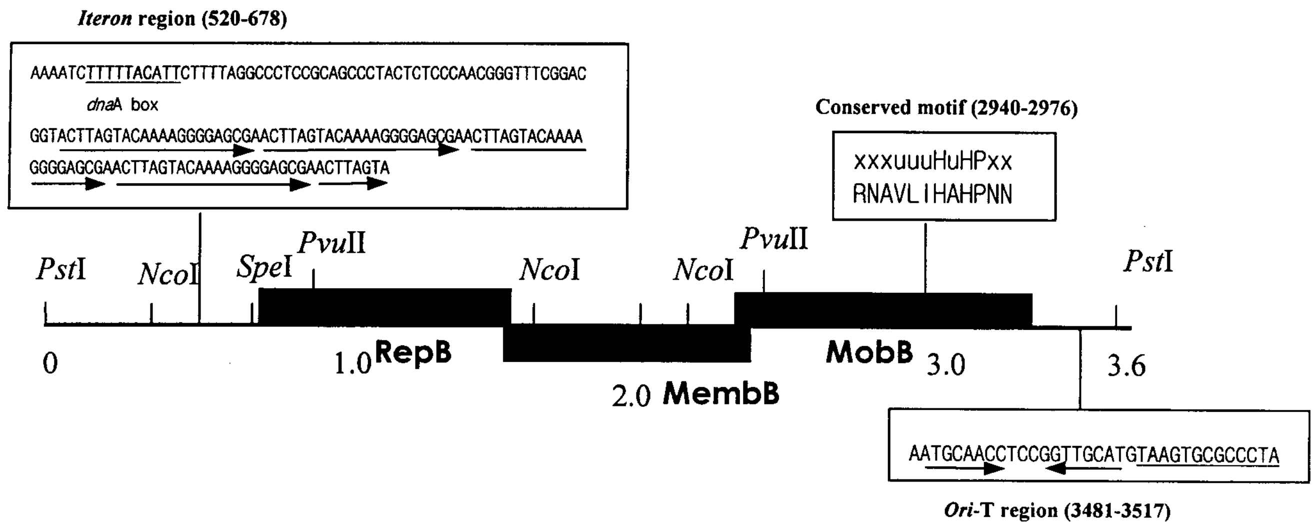


**Fig. 1.** Schematic presentation of the nucleotide sequence of pKJ36.
The numbers under the line represent the nucleotide residue in kilobases. Each ORF is indicated as a filled box. RepB and MembB is read from left to right whereas MobB reads from right to left. The nucleotide sequences of the putative DNA iteron region (520- 678) and *ori*-T region (3,481- 3,517) are shown. The amino acid sequence of the highly conserved motif (2,940- 2,976) in MobB is indicated as a box (u-hydrophobic residue, x-nonconsensus residue). This nucleotide sequence is available in the NCBI and GenBank database under accession number (AF139129).

65.1%, which was higher than that of pKJ50 with 61.7%. The sequence analysis using the DNASIS program revealed several open-reading frames (ORFs) on all six reading frames. Among them, three putative ORFs encoding basic (ORF I; pI=9.25, 32.0 kDa), highly basic (ORF II; pI= 12.25, 29.1 kDa), and another basic (ORF III; pI=10.66, 39.0 kDa) were noticed. Two of the ORFs, ORF I and ORF III, showed a high amino acid sequence homology with the RepA and MobA of pKJ50 [22], and were designated as RepB and MobB, respectively (Fig. 1).

## Gene Bank Sequence Homology Search of Putative Replication Protein, RepB

The putative RepB protein (32 kDa, pI=9.25) exhibited a relatively high amino acid sequence homology with the RepA of pKJ50 (57% amino acid identity and 70% similarity) and its homologous replication proteins of gram-positive and -negative bacteria [22]. Rep protein is generally known as the initiator of plasmid replication. When all these Rep proteins were aligned, highly conserved regions with tyrosine residues were detected (Fig. 2). These results suggest that RepA and RepB are structurally and functionally closely related, and both involved in plasmid replication and proper maintenance. Another report on the sequence analysis of a bifidobacterial plasmid has been published by Rossi *et al.* [24] who analyzed the 1,847 base pair (pMB1) plasmid from *B. longum* B2577. In this plasmid, two open reading frames were detected which showed the highest amino acid sequence similarity with the peptide sequences translated from the *C. glutamicum* pXZ10142 and *M. foruitum* pAL5000 plasmids. However, a comparison of the RepA of pKJ50 and RepB protein amino acid sequences with that of the pMB1 plasmid isolated from *B. longum* B2577 [24] exhibited no significant homology (Table 1). Moreover, there were no detectable iteron structures in the pMB1 plasmid. These results indicate that the replication mechanism of pKJ50 and pKJ36 may be different from that of pMB1.

## Replication Mechanism of pKJ36

A Southern blot analysis using a probe made of pKJ36 linearized with *Pst*I revealed that the ssDNA intermediate disappeared after treatment with endonuclease S1 (Fig. 3). This result suggests that pKJ36 produces ssDNA as the replication intermediate. Earlier, we reported that the bifidobacterial plasmid pKJ50, which is present in the same host as pKJ36, accumulated ssDNA as replication intermediates [22]. Numerous plasmids of gram-positive bacteria have also been reported to replicate *via* single-stranded intermediates, probably by a rolling circle replication (RCR) similar to the ssDNA phages of *E. coli* [13]. Generally, RC replication is dependent on three key elements encoded by the replicon itself; (i) a *cis*-acting origin (+ori) for a plus-strand synthesis, (ii) a Rep protein which nicks a unique site within the +ori region, attaches to the 5' nick terminus via a phosphotyrosine linkage, displaces the plus strands, renicks, and finally ligates at the newly generated +ori after one round of replication, and (iii) an origin for minus-strand replication (sso).

In contrast to the above results, the RepA and RepB of pKJ50 and pKJ36 showed a high amino acid sequence homology with various replication proteins from theta-replicating plasmids. This discrepancy should be clarified by further work including cloning, expression, and purification

```
RepB        IVKFSNQFNNVALKKFDAVHLDVLMAIASRVREKGTA----TVEFSFEELRGLMRLRK-------------NLTNKQLADKIVQT
RepA        IVKYSNQFNNQALRKFTALDLDLLMAIASRVRDKGTD----EVAFTFEELKRLAGLQR-------------NMTNDEFAKQIANV
gi|1072923  IVKYHNDFNKIQLPSFTEQEQNLLFLIFARIKEKGIDNVVNLYANDFKINEKLSNSKEYLTENINSLKYKFFKANFKQIIETR
gi|1072924  IVKYNNDFNLLPMPELKAIQMDMFMAIISLTKDKKEN--TPFLKKFFNPDKRKIIIPQ--KKFIELCRLNDSKMDYKEIFFAI
pHPM180     YTTYHNNANKVNLGKLSEREANLLFAIFQRLKDQGNT----LIRFEPQDLKRMIMVKSNLTNRQLLQILKNLLDNIGGANFWI
pHEI1       YTTYHNNANKVNLGKLSEREANLLFAIFQKLKDQGNT----LIRFEPQDLKRMIMVKSNLTNRQLLQILKNLLDNIGGANFWI
gi|1339881  AVKYQNELNLVPLKNFNAKQMDLFFALCARMKDKGVE----NIRFSFEELKELSDYKM-------------TATKAFVADLEKL
gi|893386   IVKYENQLNSVSFRHFNARELNLFFSIVSRMRDKGTE----KVSFTFNELHELSRYSD-------------HGERLVKDLEGV
gi|1085809  LVKYDPELNTIPLRRFTPVEMNLFFSVVSRMRDKGDD----TVRFTFDQLKELSAYKP-------------TANNRFIDDIQST
             ..:  : *   :  :   . :::: :    :::                .  .

                                                                            ↓
RepB        NARLLALNYMFEDS--GKIIQFALFTKFVTD--PQEATLAVGVNEEFAFLLNDLTSQ--FTRFELAEFADLKSKYAKEFYRRA
RepA        NRRLLALNFEFQNEE-HDIIQFALFAGFVTS--PTKRTLTVSVNSRFSFLLNDLTSQ--FTRFELAEFTALRSSYAKECYRRL
gi|1072923  TEVIHKYVNLFETME-IHYVKRNPDDGYDES--TLFNRITLKINPDFAYLVNQLTTN--FTAFELEEFIALSGKYAKTLYRLL
gi|1072924  DDCLKKLCNFLVSYQ-KDERTIYNFVCFEEAN-IIADEVHITLQSRFYDMIINKKFG--FTAFELAEFAELSGKYTKTLYRLL
pHPM180     IREHVENGEIYEDHT-SYMLFKQFDIRIHKPT-QTIEYLEVQLNDSYHYLLNNLGMGGQYTSFKLLEFQRVRGKYAKTLYRLL
pHEI1       IREHVENGEIYEDHT-SYMLFKQFDIRIHKPT-QTIEYLEVQLNDSYHYLLNNLGMGGQYTSFKLLEFQRVRGKYAKTLYRLL
gi|1339881  YKDMLNLSYRTEND--DEIEYFVLFNGFKID--KKQKFVEVRVNQQLQYIINGLTTE--FSRFELSAFTSIRSTYAKTLFRLL
gi|893386   YTKMQNLNMWYDDG--NIIEHWVLFPGFQIN--RKETTVTVSINPELKSVLNQLSNW--T-RFSLEQFASLKSTYSKTLFRLL
gi|1085809  YQKILGLRFGSRSKDGLDREMFVMFTRFEIKGSAEVPYVDIQIYPKALKLLNNLESW--V-RYALAEFRDLKSSYAKTMFRLL
                                                : : :      ::        : *  * : ..*:*  :*

                ↓
RepB        FADLKSKYAKEFYRRAKQYRSSGIWKISRDEFCRLLGVSDSTAKSTANLNRVVLKTIAEECGP
RepA        FTALRSSYAKECYRRLKQYRQTGVWKVSLEDFRRLLDVPKSYRPS--EINKYVLKPIEEELGP
gi|1072923  FIALSGKYAKTLYRLLKQFRTTGKAYFEWEEFCRIMDIPQDYRQS--EIDKRNFNQPSKNFLK
gi|1072924  FAELSGKYTKTLYRLLKQFRTTGKAYFEWEEFCRIMKIPENYRQI--DIDQRILKPAIKELSK
pHPM180     FQRVRGKYAKTLYRLLKQYKSTGILSVEWDQFRELLDIPKDYKMP--NIDKFVLKIALKELRK
pHEI1       FQRVRGKYAKTLYRLLKQYKSTGILSVEWDQFRELLDIPKDYKME--NIDQKVLTPSLKELRK
gi|1339881  FTSIRSTYAKTLFRLLMQYRSTGYYVVSIEDFRELLDIPEYYQMG--NIDQKVLKPAMKELHN
gi|893386   FASLKSTYSKTLFRLLKQYRTVGKRNFSMQEFRNLLDIPKSYSVS--DIDKKVMTPFKKELAG
gi|1085809  FRDLKSSYAKTMFRLLKQFRTTGYAYFSKSDFFELLDIPQSYWNKPANVESRVIQPIREELTP
            * : ..*:* :* *:: *  .. .:*.:: :..      ::: :   ::
```

**Fig. 2.** Multiple sequence alignment of various replication proteins (Rep proteins).

The aligned motifs are excerpts from the complete alignments generated by the CLUSTAL V program as described in Materials and Methods. The consensus line includes the amino acid residues conserved in all the aligned sequences which are marked by * and the conserved tyrosine residues are indicated by ↓.

**Fig. 3.** Southern blot analysis of the replication intermediate of pKJ36.

Single-stranded DNA (ssDNA) intermediates are indicated by the arrow (lane 1) and disappear after treatment with S1 nuclease for 15 min at 25°C (lane 2).

of each protein and a DNA binding assay with the expected replication origin.

## GenBank Sequence Homology Search of Putative Mobilization Protein, MobB

The putative MobB protein (39 kDa, pI=10.66) of pKJ36 exhibited an amino acid sequence homology with the MobA of pKJ50 (48% amino acid sequence identity and 61% similarity) and other mobilization proteins (Table 17); the nickase of the streptococcal plasmid pIP501 (30% identity, 48% similarity, [30]), traA of *Lactococcus lactis* (37% identity, 56% similarity, [8]), mobL protein of *Thiobacillus ferrooxidans* (40% identity, 49% similarity, [9]), nicking

**Table 1.** Amino acid sequence homologies and similarities with various replication proteins.

| Homologous proteins | RepA | | RepB | |
|---|---|---|---|---|
| | Homology (%) | Similarity (%) | Homology (%) | Similarity (%) |
| Rep proteins of pEFC1 from *Enterococcus faecalis* GenBank D85392 | 45 | 68 | 36 | 63 |
| RepA of pLA103 from *Lactobacillus acidophilus* | 43 | 64 | 37 | 54 |
| RepB of *Campylobacter coli* Genbank S49368 | 46 | 63 | 42 | 62 |
| RepA of *Pediococcus halophilus* Genbank S38639 | 54 | 64 | 43 | 60 |
| Rep protein of pHPM180 from *Helicobacter pylori* GenBank U12689 | 50 | 66 | 36 | 57 |
| Putative RepA of pHel 1 from *Helicobacter pylori* | 48 | 64 | 36 | 57 |
| Putative Rep protein of pMB1 from *B. longum* B2577 | N.D.* | N.D. | N.D. | N.D. |
| Putative RepA of pKJ50 from *B. longum* KJ | 100 | 100 | 57 | 70 |
| Putative RepB of pKJ36 from *B. longum* KJ | 57 | 70 | 100 | 100 |

N.D.*: Not detected.

```
RepA  MTDEIVKYSNQFNNQALRKFTALDLDLLMAIASRVRDKGTDEVAFTFEELKRLAGLQRNM 60
RepB  MSNEIVKFSNQFNNVALKKFDAVHLDVLMAIASRVREKGTATVEFSFEELRGLMRLRKNL 60
      M++EIVK+SNQFNN AL+KF A+ LD+LMAIASRVR+KGT  V F+FEEL+ L  L++N+

RepA  TNDEFAKQIANVNRRLLALNFEFQNEEHDIIQFALFAGFVTSPTKRTLTVSVNSRFSFLL 120
RepB  TNKQLADKIVQTNARLLALNYMFE-DSGKIIQFALFTKFVTDPQEATLAVGVNEEFAFLL 119
      TN + A +I   N RLLALN+ F+ +      IIQFALF  FVT P + TL V VN  F+FLL

RepA  NDLTSQFTRFELAEFTALRSSYAKECYRRLKQYRQTGVWKVSLEDFRRLLDVPKSYRPS- 179
RepB  NDLTSQFTRFELAEFADLKSKYAKEFYRRAKQYRSSGIWKISRDEFCRLLGVSDSTAKST 179
      NDLTSQFTRFELAEF  L+S YAKE YRR KQYR +G+WK+S ++F RLL V  S   S

RepA  -EINKYVLKPIEEELGPLLNLKVHRKYLKKKPGRGRASLVGFEFEFDPEKVP 230
RepB  ANLNRVVLKTIAEECGPLLGLKIERQYVKRR--------LSGFVFTFARETPP 224
       +N+ VLK I EE GPLL LK+ R+Y+K++        L GF F F  E P
```

**Fig. 4.** Amino acid sequence alignment between RepA of pKJ50 and RepB of pKJ36.
They show a 57% amino acid identity and 70% similarity.

enzyme of *Staphylococcus aureus* (31% identity, 50% similarity, [5]) and polypeptide involved in the formation of the relaxation complex of *Salmonella typhimurium* (26% identity, 42% similarity, [3]). There was also a detectable consensus motif in MobB, which was identical to that of MobA (Fig. 6). A multiple sequence alignment of these proteins also showed highly conserved domains among them (Fig. 6). One of the motifs (boxed region in Fig. 1; xxxuuuHuHPxx) was found to be identical to that previously reported as the most prominent motif among the three consensus sequences of the mobilization proteins of the pUB110 family, pMV158 family, and phage family [29].

## Hydropathy Analysis of Putative Membrane Protein, MembB

In general, segments containing about 20-30 amino acid residues with a high degree of hydrophobicity are considered to be long enough to span a membrane as an α-helix [10]. A potential transmembrane protein was detected in pKJ36 using a hydropathy plot analysis and designated as MembB. There were four possible transmembrane segments (35-55, 147-168, 219-236, and 242-262, Fig. 7) in MembB.

```
MobB       KRVHDELPEGAPAEFADPAVLFNAVELHETGRTARPAKKIVVALPREFTPRQRVQALEEYIRENLNADGYAATYAIH
MobA       ------CCPRARPASTSTRSVCSTPSRWPRNVPTRGPRRRSWSPCPASSTPANAFRALEDFISWNITANGYACTYAIH
gi|478568  EVLHAESGHMPEFVER-PADYWDAADLYER-ANGRLFKEVEFALPVELTLDQQKALASEFAQHLTGAERLPYTLAIH
gi|126781  EVLHAESGHMPEFVER-PADYWDAADLYER-ANGRLFKEVEFALPVELTLDQQKALASEFAQHLTGAERLPYTLAIH
gi|112651  EVLHAESGHMPEFVER-PADYWDAADLYER-ANGRLFKEVEFALPVELTLDQQKALASEFAQHLTGAERLPYTLAIH
gi|248708  EVLHAESGHMPEFVER-PADYWDAADLYER-ANGRLFKEVEFALPVELTLDQQKALASEFAQHLTGAERLPYTLAIH
gi|133108  DLEHKESGNMPAWAAHKPSEFWKAADTSER-ANGCTYREIEIALPRELKPEQRLELVRDFVQQEIG-DRHAYQFAIH
gi|662259  PEAFILKPDYVPNEFLDRQTLWNKMELAEKSPNAQLCREVNVALPIELNNSDQRMLIEDFVKDNFVNEGMIADVAIH
                                   .   .    :.   . * . .  :     ::      :     ***

                xxPHuHuuuxxx
MobB       E----DREGNNPHAHILVANRQIDPATGGWARLKQRMEYVLDE--------RGERVP---------------LI
MobA       T----DKDGRNPHAHILVANRRIDPKTGRWA-AKSRSEFALDA--------NGRRIP---------------VI
gi|478568  -----AGGGENPHCHLMISERINDGIERPAAQWFKRYNGKTPEKGGAQK---TEALKP----------KAWLEQTRE
gi|126781  -----AGGGENPHCHLMISERINDGIERPAAQWFKRYNGKTPEKGGAQK---TEALKP----------KAWLEQTRE
gi|112651  -----AGGGENPHCHLMISERINDGIERPAAQWFKRYNGKTPEKGGAQK---TEALKP----------KAWLEQTRE
gi|248708  -----AGGGENPHCHLMISERINDGIERPAAQWFKRYNGKTPEKGGAQK---TEALKP----------KAWLEQTRE
gi|133108  NPKAAIAGGEQPHAHIMFSERINDGIHRDPEQYFKRANTKEPD-AVAQKRHVSGKHRPN--------AKNTLLPRGR
gi|662259  -----RDDENNPHAHIMLTMREVDSEGNILNKSHRIPKLDENGNQIFNE---KGQRVTVSIKTNDWGRKSLVSEIRK
                  .:**.*::.: *  *           :              .

MobB       DPETGRQKTDKRG-------RRQWKRTSVSLNPLDRKAKLKALR-------ESWAKTCNARLDETARIDHRSLED--
MobA       DPDTGRQKIGARN--------RKVWKRVNVSNNPLDSKEFLERLR-------REWADSCNALLPGYAVIDHRSFKA--
gi|478568  AWADHANRALERAGHDARIDHRTLEAQGIERLPGVHLGPNVVEMEGRGIRTDRADVALNIDTANAQIIDLQEYR--
gi|126781  AWADHANRALERAGHDARIDHRTLEAQGIERLPGVHLGPNVVEMEGRGIRTDRADVALNIDTANAQIIDLQEYR---
gi|112651  AWADHANRALERAGHDARIDHRTLEAQGIERLPGVHLGPNVVEMEGRGIRTDRADVALNIDTANAQIIDLQEYR---
gi|248708  AWADHANRALERAGHDARIDHRTLEAQGIERLPGVHLGPNVVEMEGRGIRTDRADVALNIDTANAQIIDLQEYR--
gi|133108  RWADLQNKHLERYQHADRVDSRSLKAQGIDREPERHLGAGQVQR----FDTEQLQAILERREAERQVQQSRDERD--
gi|662259  DWADKVNQYLKDRNIDQQITEKSHAELGKKELPTIHEGFYSKKLEDKGVISELKRKNLEIQSYNDILAELDKLENQE
             ::         :     . .  *                          :         :  . .
```

**Fig. 5.** Multiple alignments of various Mob proteins.
The aligned motifs are excerpts from the complete alignments generated by the CLUSTAL V program as previously described. The consensus line includes the amino acid residues conserved in all the aligned sequences which are marked by *.

In contrast to the acidic properties of MembA of pKJ50 [22], MembB exhibited an extremely basic calculated pI value (12.25). The amino acid sequence homology search

**Table 2.** Amino acid sequence homologies and similarities with various plasmid mobilization proteins.

| Homologous proteins | MobA | | MobB | |
|---|---|---|---|---|
| | Homology (%) | Similarity (%) | Homology (%) | Similarity (%) |
| MobA of *E. coli* plasmid RSF1010 | 55 | 69 | 31 | 43 |
| 43 kDa relaxation protein from pSC101 of *Salmonella typhimurium* | 55 | 70 | 26 | 42 |
| Nicking enzyme of pGO1 from *Staphylococcus aureus* | 35 | 60 | 31 | 50 |
| TraA of pTiC58 from *Agrobacterium tumefaciens* | 37 | 68 | 33 | 53 |
| Putative MobA of pKJ50 from *B. longum* KJ | 100 | 100 | 48 | 61 |
| Putative MobB of pKJ36 from *B. longum* KJ | 48 | 61 | 100 | 100 |

```
MobA   PRARPASTSTRSVCSTPSRWPRNVPTRGPRRRSWSPCPASSTPANAFRALEDFISWNITA 86
MobB   PEGAPAEFADPAVLFNAVELHETGRTARPAKKIVVALPREFTPRQRVQALEEYIRENLNA 115
       P   PA  +  +V              T  P ++     P   TP    +ALE++I   N+ A

MobA   NGYACTYAIHTDKDGRNPHAHILVANRRIDPKTGRWAA-KSRSEFALDANGRRIPVIDPD 145
MobB   DGYAATYAIHEDREGNNPHAHILVANRQIDPATGGWARLKQRMEYVLDERGERVPLIDPE 175
       +GYA TYAIH D++G NPHAHILVANR+IDP TG WA  K R E+ LD  G R+P+IDP+

MobA   TGRQKIGARNRKVWKRVNVSNNPLDSKEFLERLRREWADSCNALLPGYAVIDHRSFKARG 205
MobB   TGRQKTDKRGRRQWKRTSVSLNPLDRKAKLKALRESWAKTCNARLDETARIDHRSLEDQG 235
       TGRQK   R R+ WKR +VS NPLD K  L+ LR  WA +CNA L   A IDHRS + +G

MobA   IERIPTIHEGYASREMEKRGGRGDLCEENRRIQALNRLLDALRAMIGRLSDQAQGILTAV 265
MobB   SDLEPTIHEGYAARAIERAGGVSERCEANREIRRSNGLLTAIRTELGRIFDRL-GELFAA 294
        +  PTIHEGYA+R +E+ GG  + CE NR I+  N LL A+R  +GR+ D+  G L A

MobA   KRR 268
MobB   KIR 297
       K R
```

**Fig. 6.** Amino acid sequence alignment between MobA of pKJ50 and MobB of pKJ36.

They show a 48% amino acid identity and 61% similarity.

of the 91-168 segments showed a 23% identity with the surface protein of *Paramecium tetraurelia* [21]. The highly basic structure of membB would seem to suggest that it interacts with the highly acidic membA protein, thereby playing a role in the mobilization of the conjugating plasmid through temporarily binding to the plasmid DNA. When the functions of these putative proteins are elucidated by using this antibody of them, they could be used as a membrane anchor for the cell surface presentation of genetically engineered antigen molecules for live vaccine.

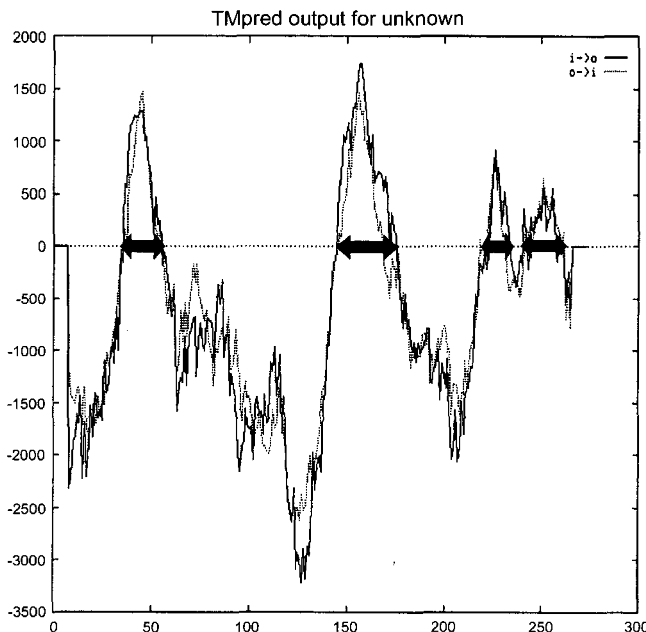


**Fig. 7.** Hydropathy plot analysis of putative Memb B protein.

The dashed and dotted lines show two different possibilities. The arrows indicate the four putative trans-membrane regions (35-55, 147-168, 219-236, and 242-262).

## DNA Iteron Sequence of pKJ36

In the upstream of *repB*, an iteron structure was found which repeated tandemly four-and-a-half times with a 22 bp unit (583-678, Fig. 1). DNA iteron is known to act as a binding site for the Rep protein and it may also control the plasmid copy number and incompatibility [12]. All of the identified replicons from theta-type plasmids, such as the pCI305/pWV02 family of *Lactococcus*, contain the tandem-repeating sequences found in the *repA* locus of pCI305, however, the repeat sequences are variable [11, 15, 26]. A 10-bp sequence matching the consensus sequence for a *dnaA* box (TTTTTACATT), as defined by Schaefer and Messer [28], was found upstream from the iterons (Fig. 1), which is a common feature for iteron-containing plasmids [16]. DNA iterons vary in their sequence, length, and number between different bacterial species and plasmids, while also playing the role as the main incompatibility determinant of a plasmid [7]. The two iterons of pKJ50 and pKJ36 in the same host were different in their sequences and structures, which may establish the compatibility between them. We are now in a

```
pKJ50        TGAATGTTACCACCGGTAACATGTAAGTGCGCCCTCAAT
              -  ------->   <------- -
pKJ36          AATGCAACCTCCGGTTGCATGTAAGTGCGCCCTAATC
               ------->   <-------
pIP501     ATACGAAGTAACGAAGTTACTGCGTATAAGTGCGCCCTTAGT
            ------ ---->   <--- -------
RSF1010      CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCCC
              -- ----->    <----- --
pSC101          TGAACGAAGTGAAGAAAGTCTAAGTGCGCCCTGAT

pTF1         GGGTAATCTCGAAGAGATTACTCTAAGTGCGCCCTTGC
              - ------->   <------ -
pGO1           CACGCGAACGGAACGTTCGCATAAGTGCGCCCTTAC
                 ------>   <------
R1162        CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCC
             -- ------>   <------ --
pTiC58       CAAGGCGTCGCGTCAGCGACGTATAATTGCGCCCTTG
                 ----->   <-----
                                  *** *********
```

**Fig. 8.** The *oriT*-like regions of pKJ50 and pKJ36.

The molecular arrangement of the *oriT* regions of pKJ50 and pKJ36 is shown at the top. The putative *oriT* site is found upstream of each mob gene. The nucleotide sequence of the putative *oriT* sites of pKJ50 and pKJ36 aligned with similar sequences found in various other plasmids, pIP501, RSF1010, pTF1, pSC101, pGO1, R1162, and pTiC58, are shown below. The palindrome sequences are marked by dashed lines and converging arrows. The asterisks and bold characters indicate the conserved sequences among these eight plasmids.

process of carrying out deletion and mutational analyses to understand the role of the iteron structure on the copy number control of these two plasmids.

### *oriT* Sequence of pKJ36

An another interesting aspect was the presence of a putative *oriT* site in the pKJ36 DNA with an identical 12 bp sequence (Fig. 8) with that found in pKJ50, the gram-negative plasmids RSF1010, pTF1, pSC101, and R1162, and the recently published *oriT* sequences of the streptococcal plasmid pIP501 [30], staphylococcal plasmid pGO1 [5], and *A. tumefaciens* plasmid pTiC58 [6]. The inverted repeat sequence commonly found in the upstream region of the *oriT* sequence was also present in pKJ36. These results suggest the possibility of gene transfer mechanisms via bacterial conjugation in the genus *Bifidobacterium.* However, no evidence to support this has yet been reported.

### *In vitro* Transcription/Translation of RepB, MobB, and MembB Proteins of pKJ36

The sequence analysis of pKJ36 suggested that this plasmid seemed to have three ORFs, as described above. A coupled *in vitro* transcription/translation reaction using several DNA templates, which contained pKJ36 fragments and $^{35}$S-methionine as a chase material, confirmed this suggestion. The promoter of each ORF on pKJ36 was used for *in vitro* transcription/translation. When pKJ36 was used as the DNA template, the *in vitro* translation reaction produced three major protein bands positioned at 32, 29, and 39 kDa, which corresponded to the putative RepB, MembB, and MobB proteins, respectively (Fig. 9). These results established that the sequence analysis of pKJ36 was correct.

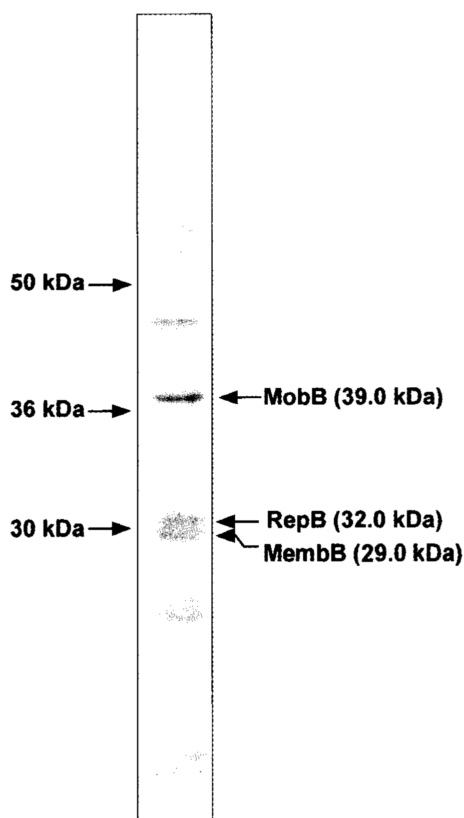


**Fig. 9.** Coupled *in vitro* transcription/translation of each ORF on pKJ36.
The proteins were expressed as described in Materials and Methods using $^{35}$S-methionine as a chaser and pKJ36 as the DNA template. The proteins were resolved by SDS-PAGE (15% acrylamide) and visualized by autoradiography.

### RT-PCR of *repB* and *membB* Genes

The RT-PCR technique was used to detect the mRNA that might be transcribed from each ORF on pKJ36, as expected from the DNA sequence analysis. By using three primer sets specific for the up and down stream sequences of each ORF of pKJ36 (Materials & Methods), the expected PCR product with the expected size was detected with respect to RepB (750 bp) and MembB (840 bp in Fig. 10). The size of each RT-PCR product was identical to those of the PCR products produced using the same primer sets and pKJ36 plasmid DNA. As in the case of the MobA of pKJ50, MobB showed no RT-PCR product although the PCR reaction with the pKJ36 template produced the expected products. This would suggest that the Mob protein in *B. longum* KJ is not usually expressed. Accordingly, the expression of the Mob protein may be controlled by an unknown mechanism and only expressed during the transfer of the plasmid between bifidobacterial strains.

### Shuttle Vector Construction Using pKJ36

A vector construction scheme similar to that of pKJ50 was applied to pKJ36. The entire pKJ36 was linearized with *Pst*I

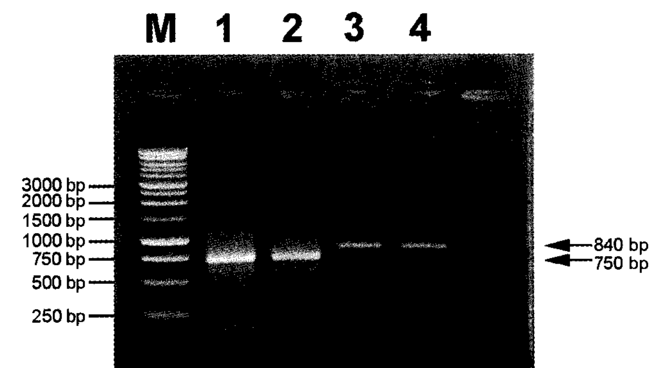


**Fig. 10.** Confirmation of the expression of each ORF of pKJ36 by RT-PCR and PCR.
The PCR and RT-PCR products were produced as described in Materials and Methods. Lane 1: PCR product with pKJ36 template and primers DW001 and DW002; Lane 2: RT-PCR product with total RNA from *B. longum* KJ and primers DW001 and DW002; Lane 3: PCR product with pKJ36 template and primers MS015 and MS016; Lane 4: RT-PCR product with total RNA from *B. longum* KJ and primers MS015 and MS016.

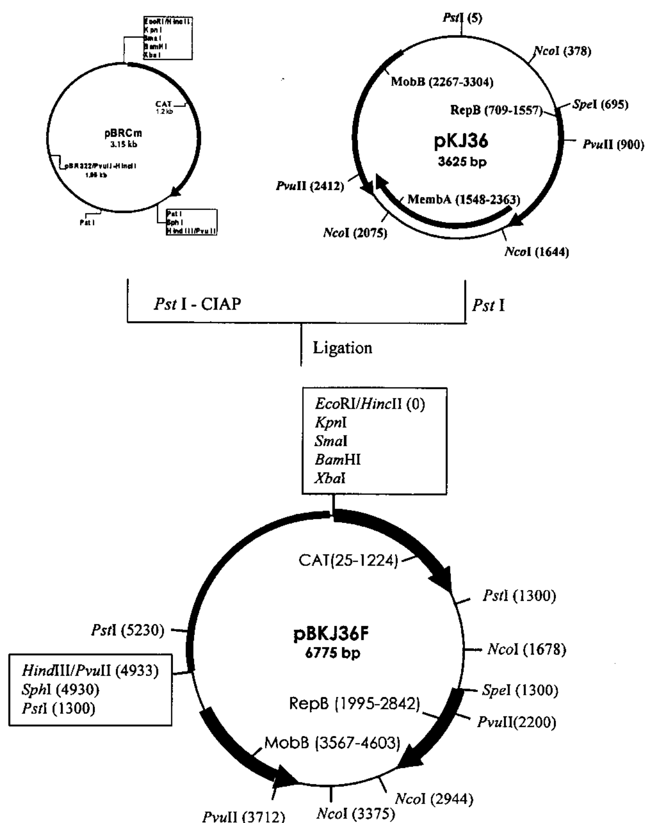


**Fig. 11.** Restriction enzyme digestion map of pKJ36 and construction of shuttle vectors.
The two shuttle vectors (pBKJ36F and pBKJ36R) are differentiated by the forward and reverse directions of the inserted pKJ36 (1300- 4925).

and then ligated into the *Pst*I site of pBRCm to construct pBKJ36F and pBKJ36R according to their orientation (Fig. 11). The transformation efficiency was similar to that of pBKJ50. The successful transformation of pBKJ36 was confirmed by the PCR amplification of the *repB* gene from the transformants (Fig. 12). The transformation efficiency differed from strain to strain and depending on the electroporation conditions with a range between $1.2 \times 10$ (*B. animalis* MB209) - $2.6 \times 10^2$ (*B. infantis* ATCC27920) cfu/mg DNA.

In this report, the full sequence and structure of pKJ36 were analyzed, and an *in vitro* translation and transcription confirmed the putative gene products. In addition, pKJ36 was compared with pKJ50 that resides in the same host.

The replication and mobilization proteins of pKJ36 were highly homologous with those of pKJ50 and those from phylogenetically distant microorganisms, whereas they did not show any homology with those of the previously characterized pMB1 of *B. longum* B2577 and pAP1 of *B. asteroides* (Genbank Y11549). Although the bifidobacteria and other bacteria with homologies were phylogenetically distant, most of the microorganisms are commonly found in the human intestine. This result suggests that there may

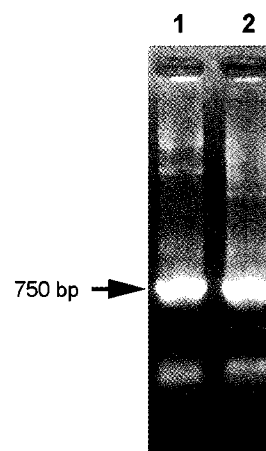


**Fig. 12.** Confirmation of transformation of *B. animalis* MB209 with constructed shuttle vector pBKJ36F by PCR.
The PCR reaction was performed using specific primers for the *rep*B gene (DW001 and DW002). The PCR products are indicated by an arrow. Lane 1: PCR product from pKJ36; Lane 2: PCR product from total DNA of the MB209 transformant with pBKJ36

be some plasmid exchange among those microorganisms. Characteristically, pKJ50 and pKJ36 carried highly acidic and basic membrane proteins, respectively. It would be interesting to determine whether the basic membB and acidic membA interact with each other and also whether membB is involved in the plasmid transfer of an acidic DNA molecule. Accordingly, the transmembrane characteristics of MembA and MembB could be utilized for the targeting of genetically engineered foreign proteins into a membrane to construct live vaccine. Finally, the use of the characteristics of this plasmid may result in more improved cloning vectors which can express a foreign gene into *Bifidobacterium.*

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