

Sequence analysis of plasmid pKJ50 from *Bifidobacterium longum*

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The complete nucleotide sequence of a plasmid, pKJ50, isolated from an intestinal bacterium, *Bifidobacterium longum* KJ, has been determined. The plasmid was analysed and found to be 4960 bp in size with a G+C content of 61.7 mol%. Computer analysis of sequence data revealed three major ORFs encoding putative proteins of 31.5 (ORFI), 24.5 (ORFII) and 38.6 kDa (ORFIII). ORFI encodes a protein with a pI of 10.18 and shows relatively high amino acid sequence similarity (more than 60%) with several plasmid replication proteins from Gram-positive and -negative bacteria. Southern blot analysis showed that pKJ50 accumulates an ssDNA intermediate, suggesting that it replicates by a rolling-circle mechanism. Upstream of ORFI, three sets of repeated sequences resembling iteron structures of related plasmids were identified. ORFIII encodes a protein with a pI of 10.97. It also shows a high level of amino acid sequence similarity with some plasmid mobilization proteins. Upstream of ORFIII, a 12 bp stretch resembles an *oriT* DNA sequence with inverted repeats identical to those found in conjugative plasmids. Hydropathy plot analysis of ORFII, encoding an acidic protein (pI = 4.95), suggests it is a transmembrane protein. Several interesting palindromic sequences, repeat sequences and hairpin-loop structures around ORFI, which might confer regulatory effects on the replication of the plasmid, were also noted. Reverse transcriptase PCR (RT-PCR) and *in vitro* translation confirmed the expression of ORFI and ORFII. RT-PCR produced amplified DNA fragments of the expected sizes, corresponding to ORFI and ORFII. However, no RT-PCR product corresponding to ORFIII was obtained. *In vitro* translation showed protein bands of the expected sizes, corresponding to each ORF. A shuttle vector capable of transforming *Bifidobacterium animalis* MB209 was constructed by cloning pKJ50 and a chloramphenicol resistance gene into pBR322.

Keywords: *Bifidobacterium longum*, plasmid, gene expression, shuttle vector, replication

INTRODUCTION

Bifidobacteria were first discovered in 1899 by Tissier at the Institut Pasteur, Paris. They are considered to play an important role in the proper balance of normal intestinal flora and exert many beneficial effects on the health of human beings. This may arise as a consequence of altered intestinal pH, specifically through the release of acetic and lactic acids. Also, the contribution of

bifidobacteria to the reinforcement of host immune functions and improved resistance to cancer have been reported (Lee *et al.*, 1993). As a consequence, biotechnological development of bifidobacteria probiotic strains with improved characteristics is becoming popular. To develop a cloning vector for modification of bifidobacteria, a comprehensive understanding of the replication mechanism and characterization of bifidobacteria plasmids is necessary. However, problems may arise as a consequence of the difficulties of isolation and cultivation of bifidobacteria. Only a few studies have been published concerning the isolation and characterization of plasmids from bifidobacteria (Bourget *et al.*, 1993; Matteuzzi *et al.*, 1990; Sgorbati *et al.*, 1986)

Abbreviation: RT-PCR, reverse transcriptase PCR.

The GenBank accession number for the sequence reported in this paper is U76614.

and vector construction using *Bifidobacterium* plasmids (Argnani *et al.*, 1996; Missich *et al.*, 1994). Recently, the sequence of the 1847 bp plasmid from *Bifidobacterium longum* B2577, whose ORF showed amino acid sequence homology with peptides from pXZ10142 of *Corynebacterium glutamicum* and pAL5000 of *Mycobacterium fortuitum*, was reported (Rossi *et al.*, 1996).

In this study, we report the full sequence of pKJ50, one of the two plasmids that exist in *B. longum* KJ (Park *et al.*, 1997). The analysis of gene structure revealed that the replication protein (Rep; ORFI) and mobilization protein (Mob; ORFIII) of pKJ50 are highly homologous with those of various Gram-positive and -negative bacteria. However, the Rep protein of pKJ50 shows little homology with that of pMB1 from *B. longum* B2577. Moreover, putative Mob and membrane proteins have been identified and structurally analysed. The replication mechanism of pKJ50 was investigated by S1 nuclease treatment and Southern hybridization. Reverse transcriptase PCR (RT-PCR) and *in vitro* translation experiments confirmed the expression of each ORF. In addition, a shuttle vector capable of transforming *Escherichia coli* and *Bifidobacterium animalis* MB209 was constructed.

METHODS

Bacterial strains, media and plasmids. *E. coli* DH5 α , cultured at 37 °C in Luria broth with vigorous shaking, was used for transformation. Ampicillin was used at a concentration of 50 μ g ml⁻¹. *Bifidobacteria* were cultured in MRS broth (Difco) supplemented with 0.05% (final concentration) cysteine/HCl at 37 °C. Multicopy plasmid vector pUC19 was used for cloning pKJ50. *B. animalis* MB209 (kindly provided by Dr Diego Matteuzzi, Italy) was used as the transformation host for the constructed shuttle vector.

General cloning techniques and sequence analysis. Small- and large-scale plasmid DNA preparation from *E. coli*, restriction enzyme digestion, ligation and transformation of *E. coli* was carried out according to the procedures of Sambrook *et al.* (1989). Plasmid DNA from *Bifidobacterium* strains was prepared as described by Park *et al.* (1997). DNA was recovered from agarose gels by using the GeneClean Kit (Bio101). For the sequencing reaction, plasmid DNA was prepared from *E. coli* DH5 α with the Wizard Minipreps DNA Purification System (Promega), according to the manufacturer's instructions. Unidirectional deletion mutants were constructed with the Kilo-Sequencing Deletion Kit (Takara Shuzo). Sequencing reactions were performed using the Cy5 AutoRead Sequencing Kit (Pharmacia) and ALF DNA Sequencer (Pharmacia). DNA and amino acid sequence data analyses were performed using the DNASIS and PROSIS programs (Hitachi Software Engineering), respectively. DNA and amino acid sequence homology searches were done online using the BLAST server maintained at the National Center for Biotechnology Information, Bethesda, MD, USA (<http://www.ncbi.nlm.nih.gov/Recipon/blast-search.html>) and the programs BLASTN, BLASTP, BLASTX and TBLASTN (Altschul *et al.*, 1990). Multiple sequence alignments of related amino acid sequences were performed using CLUSTAL V (Higgins, 1994). Hydropathy plot analysis of each deduced amino acid was done using an Internet program (<http://www.microbiology.adelaide.edu.au/leam/index.htm>).

Southern blot analysis and detection of ssDNA. Southern blot analysis was performed using a digoxigenin (DIG) DNA Labelling and Detection Kit (Boehringer Mannheim), according to manufacturer's protocol. ssDNA was detected by the method of Leenhouts *et al.* (1991), which relies on comparing plasmid DNA before and after selective digestion of ssDNA with S1 endonuclease.

Total RNA isolation from *B. longum* KJ and RT-PCR. Total RNA was isolated using the High Pure RNA Isolation Kit (Boehringer Mannheim). *B. longum* KJ was cultivated to mid-exponential phase (OD₆₀₀ = 0.5). From this culture, about 1 × 10⁹–1 × 10¹⁰ c.f.u. ml⁻¹ were harvested by centrifugation at 2000 g for 5 min and the pellet was resuspended in 200 μ l 10 mM Tris/HCl (pH 8.0) supplemented with 4 μ l lysozyme solution (50 mg ml⁻¹) and incubated for 10 min at 37 °C. Four hundred microlitres of lysis/binding buffer (4.5 M guanidine/HCl, 50 mM Tris/HCl, 30% Triton X-100, pH 6.6) was added to the solution, mixed well and transferred to the upper reservoir of a High Pure filter tube. After centrifugation at 8000 g for 15 s to discard the flow-through, 100 μ l DNase I solution was added to the tube and incubated for 60 min at room temperature. It was washed twice with wash buffer I (5 M guanidine/HCl, 20 mM Tris/HCl, pH 6.6) and buffer II (20 mM NaCl, 2 mM Tris/HCl, pH 7.5) by centrifugation at 8000 g for 15 s. Then the filter tube was transferred to a sterile microcentrifuge tube and the RNA was eluted with 50 μ l elution buffer (nuclease-free, sterile double-distilled water) by centrifugation at 8000 g for 1 min. The RNA solution was aliquoted and stored at -70 °C. RT-PCR was performed using the Access RT-PCR system (Promega), according to the manufacturer's protocol. The synthesis of the first cDNA strand was carried out using specific primers [for ORFI, MS005 (5' CAGTCATGACCGATGAGATC 3') and MS006 (5' GAGTATCAGGGTTCGAGCGCC 3'); for ORFII, MS001 (5' CGGGATGCATGACTTTTCG 3') and MS002 (5' ATCCTTACCAGCGGGTAACG 3'); for ORFIII, MS003 (5' TCAAGCCGTGTCTTGAACCT 3') and MS004 (5' CGC-GTCGAGCATGTATGCAC 3')] and AMV reverse transcriptase (Promega) at 48 °C for 45 min. PCR was performed by using the same primers and *Taq* DNA polymerase (Promega) for 40 cycles as follows: denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and elongation at 68 °C for 2 min. This was followed by one final extension step at 68 °C for 7 min.

Coupled *in vitro* transcription/translation. *In vitro* translation was performed using the *E. coli* T7 S30 extract system for circular DNA (Promega), according to the manufacturer's protocol. Four micrograms of template DNA, 5 μ l amino acid mixture without methionine, 20 μ l S30 premix without amino acids, 1 μ l ³⁵S-methionine (4.55 × 10¹³ Bq ml⁻¹) and 15 μ l T7 S30 extract were mixed gently, sterilized double-distilled water was added to 50 μ l and the mixture was incubated at 37 °C for 2 h. The reaction was stopped by placing the tube on ice for 5 min. Five microlitres of reaction mixture was transferred to a new microcentrifuge tube and 20 μ l acetone was added and mixed. After 15 min incubation on ice, the mixture was centrifuged at 12000 g and the supernatant was discarded and dried for 15 min. The pellet was resuspended in 20 μ l SDS sample buffer and boiled at 100 °C for 3 min. It was analysed by conventional SDS-PAGE (15% acrylamide) and visualized by autoradiography.

Detection of ssDNA replication intermediate. To detect the replication intermediate, Southern blot analysis was performed according to the method of Leenhouts *et al.* (1991).

Shuttle vector construction and transformation of *B. animalis*

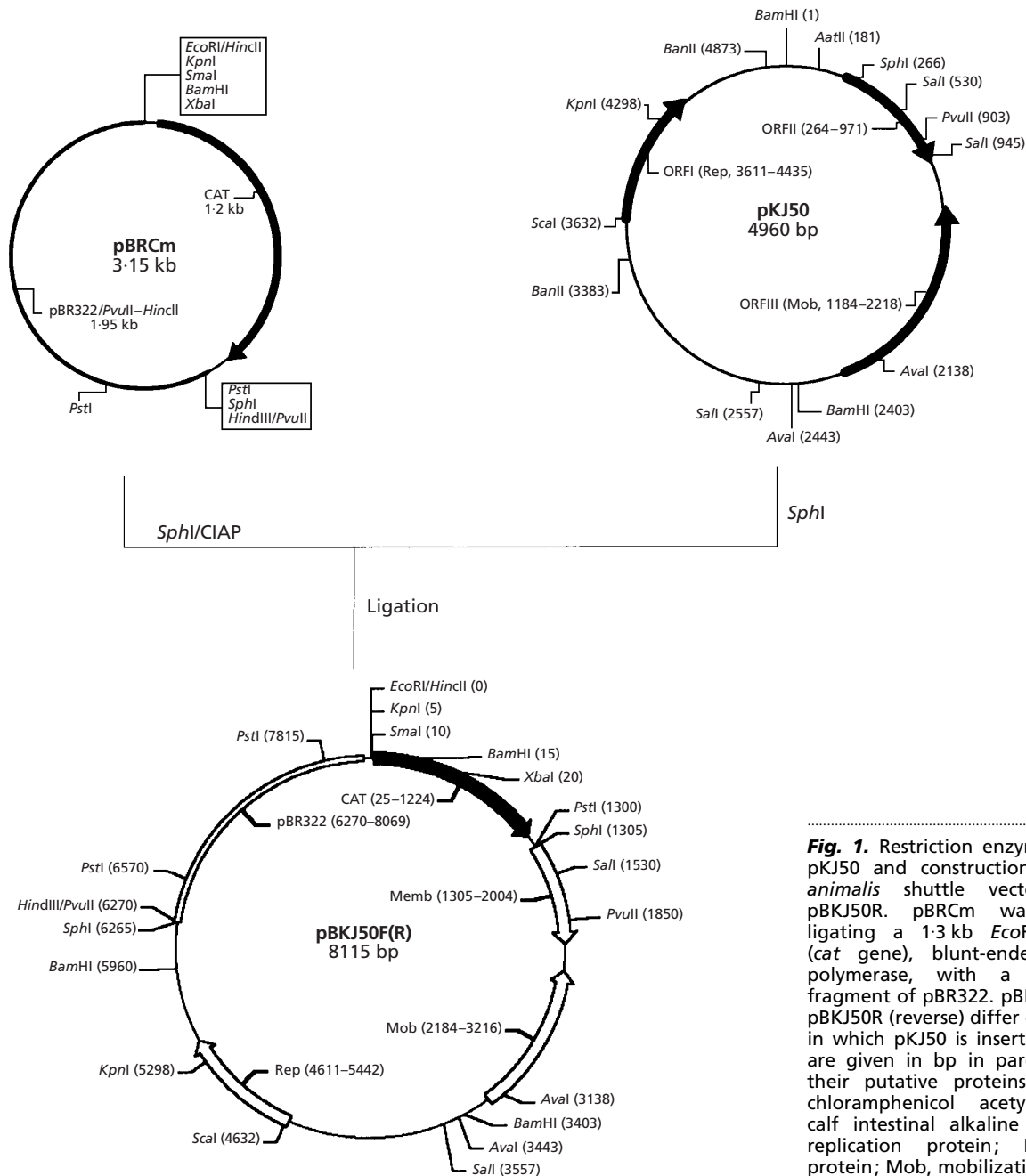


Fig. 1. Restriction enzyme map of plasmid pKJ50 and construction of the *E. coli*-*B. animalis* shuttle vectors pBKJ50F and pBKJ50R. pBRCm was constructed by ligating a 1.3 kb *EcoRI*-*HindIII* fragment (*cat* gene), blunt-ended using T4 DNA polymerase, with a 1.8 kb *PvuII*-*HincII* fragment of pBR322. pBKJ50F (forward) and pBKJ50R (reverse) differ only in the direction in which pKJ50 is inserted. Site coordinates are given in bp in parentheses. ORFs and their putative proteins are shown: CAT, chloramphenicol acetyltransferase; CIAP, calf intestinal alkaline phosphatase; Rep, replication protein; Memb, membrane protein; Mob, mobilization protein.

MB209. For the construction of the shuttle vector, the 1.3 kb staphylococcal chloramphenicol acetyltransferase gene (*cat*) (*EcoRI*-*HindIII* fragment of pEK104; Park *et al.*, 1989) was first ligated with the 1.8 kb pBR322 *PvuII*-*HincII* fragment and designated pBRCm (Fig. 1). The *cat* gene enabled *E. coli* transformants carrying pBRCm to grow in the presence of 25 μ g chloramphenicol ml^{-1} . Then, *E. coli*-*B. animalis* shuttle vectors pBKJ50F and pBKJ50R were constructed by cloning the whole pKJ50 plasmid into the *SphI* site of pBRCm as shown in Fig. 1. Electrocompetent cells of *B. animalis* MB209 were prepared according to the method of Argnani *et al.* (1996). The pulse generator of EasyJect One (EquiBio) was set at 10 kV cm^{-1} , 200 Ω and 40 μF .

RESULTS AND DISCUSSION

DNA sequence of pKJ50

Plasmid pKJ50 was originally isolated from *B. longum* KJ screened from the faeces of an infant by Park *et al.* (1997). After its initial isolation, *B. longum* KJ was subcultured more than 100 times under non-selective conditions without losing its plasmid, indicating that replication of pKJ50 in this strain is extraordinarily stable under laboratory conditions. For the structural analysis of pKJ50 in this study, it was subcloned and fully sequenced as follows. pKJ50 was linearized with

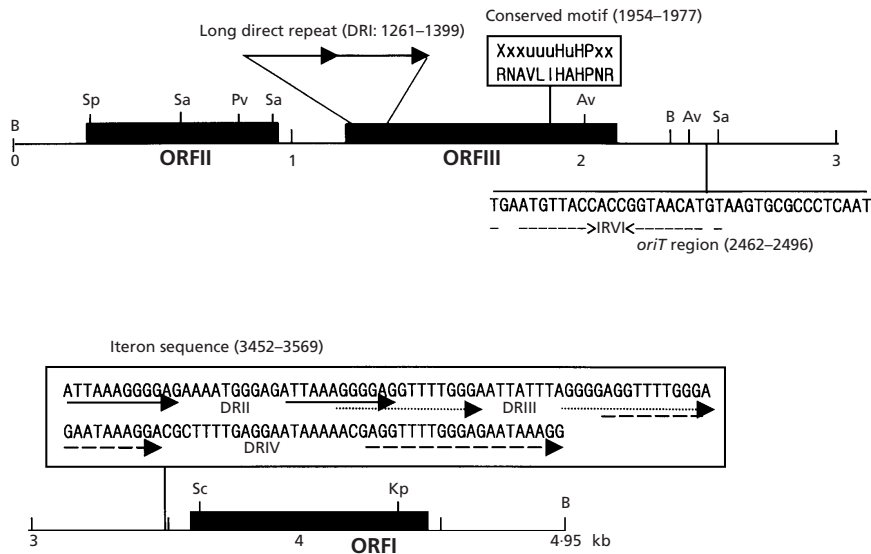


Fig. 2. Schematic representation of the nucleotide sequence of pKJ50. Each ORF is indicated by a black box. ORFI and ORFII read from left to right and ORFIII reads from right to left. The nucleotide sequences of the putative DNA iteron region (3452-3569) and the *oriT* region (2462-2496) are shown. The amino acid sequence of the highly conserved motif (1954-1977) in ORFIII is indicated in a box (u, hydrophobic residue; x, non-consensus residue). Abbreviations: Av, *Aval*; B, *BamHI*; Kp, *KpnI*; Pv, *PvuII*; Sa, *SalI*; Sc, *Scal*; Sp, *SphI*.

KpnI and cloned into the *KpnI* site of pUC19 to construct pMS50. The *BamHI* fragments of pMS50 were subcloned into the pUC19 *BamHI* site to produce p3400 (self-ligation of the 3.4 kb *BamHI* fragment), p51 and p51R (containing the 2.4 kb *BamHI* fragment in both forward and reverse directions), and p46 and p46R (containing the 1.9 kb *BamHI* fragment in both directions). Deletion mutants were prepared and sequenced on both strands. A diagrammatic representation of the nucleotide sequence of pKJ50 is shown in Fig. 2 [numbering starts at *BamHI* site 1 (Fig. 1)]. The G + C content is 61.7 mol%, slightly higher than that of the genome (60.1 mol%; Bezkorovainy & Miller-Catchpole, 1989) of *Bifidobacterium* sp. There are three putative ORFs encoding basic (ORFI, pI = 10.18, 31.5 kDa), acidic (ORFII, pI = 4.95, 24.5 kDa) and basic (ORFIII, pI = 10.97, 38.6 kDa) proteins. These ORFs are indicated in the restriction map of pKJ50 (Fig. 1).

Replication by a rolling-circle mechanism

There is no report of the replication mechanisms of bifidobacteria plasmids to our knowledge. To examine if *Bifidobacterium* plasmids replicate via the rolling-circle mechanism, accumulation of ssDNA intermediates in plasmid-harboring cells was examined. Plasmid DNA was isolated from *B. longum* KJ and divided into two portions, one of which was treated with endonuclease S1 to digest ssDNA. Southern blot analysis of the S1-digested and non-digested plasmid DNA fractions, using pKJ50 linearized with *KpnI* as probe, revealed that the ssDNA intermediate disappeared after treatment with endonuclease S1 (Fig. 3). This suggests that pKJ50 produces ssDNA as a replication intermediate. Numerous plasmids of Gram-positive bacteria have been reported to replicate via single-stranded intermediates, probably by rolling-circle replication similar to the ssDNA phages of *E. coli* (Gruss & Ehrlich, 1989).

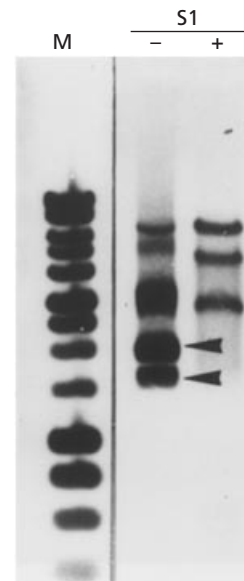


Fig. 3. Detection of the ssDNA replication intermediate of pKJ50. ssDNAs are indicated by arrows. Plasmid DNAs were isolated from *B. longum* KJ and divided into two portions, one of which was treated with endonuclease S1 to digest ssDNA (lane +). Southern blot analysis using pKJ50 linearized with *KpnI* revealed that the ssDNA intermediate disappeared after treatment with endonuclease S1. Lane M, molecular mass markers.

Replication protein coding region

The deduced amino acid sequence of the pKJ50 Rep protein resembles that of the Rep proteins of pEFC1 from *Enterococcus faecalis* (45% identity, 68% similarity; GenBank D85392), RepA of pLA103 from *Lactobacillus acidophilus* (43% identity, 64% similarity; Kanatani *et al.*, 1995), RepB of *Campylobacter coli* (46% identity, 63% similarity; GenBank S49368), RepA of *Pediococcus halophilus* (54% identity, 64% simi-

larity; GenBank S38639), the 54.52 kDa protein of pHPM180 from *Helicobacter pylori* (50% identity, 66% similarity; GenBank U12689) and the putative RepA of pHeI1 from *H. pylori* (48% identity, 64% similarity; Heuermann & Haas, 1995). Multiple amino acid sequence alignment analysis showed highly conserved regions between these sequences (data not shown). Regions upstream of ORFI contain an AT-rich sequence followed by three sets of direct repeat sequences flanking each other (DRII, DRIII and DRIV, Fig. 2). This motif resembles the so-called DNA iteron structure, a feature typical of the *ori* site of many bacterial plasmids. These motifs act as binding sites for the Rep protein and may control plasmid copy number and incompatibility (Frey *et al.*, 1992). All of the identified replicons of theta-type plasmids, such as the pCI305/pWV02 family of *Lactococcus*, contain the tandem repeat sequences found in the *repA* locus of pCI305; however, the sequences of the repeats are variable (Frere *et al.*, 1993; Kiewiet *et al.*, 1993; Seegers *et al.*, 1994). No conserved regions encoded by replication proteins involved in the rolling-circle mechanism (Wang & Macrina, 1995) were observed in pKJ50. Comparison of the pKJ50 Rep protein amino acid sequence with that of plasmid pMB1 from *B. longum* B2577 (Rossi *et al.*, 1996) and pAP1 from *B. asteroides* (GenBank Y11549) showed no significant homology.

Mobilization protein coding region

ORFIII, encoding a putative 38.6 kDa protein with a pI of 10.97, showed significant amino acid sequence homology with some mobilization proteins, e.g. MobA of *E. coli* plasmid RSF1010 (55% identity, 69% similarity; Drolet *et al.*, 1990; Frey *et al.*, 1992) and the 43 kDa relaxation protein from pSC101 (55% identity, 70% similarity; Drolet *et al.*, 1990) of *Salmonella typhimurium*. It also shared amino acid sequence homology with the nicking enzyme of pGO1 from *Staphylococcus aureus* (35% identity, 60% similarity; Climo *et al.*, 1996) and TraA of pTiC58 from *Agrobacterium tumefaciens* (37% identity, 68% similarity; Cook & Farrand, 1992). The sequence alignment of these proteins also showed highly conserved domains (Fig. 4). One of those motifs (boxed region in Fig. 4) is identical to that previously reported by Tatyana & Eugene (1992) as the most prominent among three consensus sequences of the mobilization proteins of the pUB110 family, the pMV158 family and the phage family. Another interesting aspect was the presence of a putative *oriT* site in pKJ50 with an identical 12 bp sequence found in Gram-negative plasmids RSF1010, pTF1, pSC101 and R1162 as well as the published *oriT* sequence of streptococcal plasmid pIP501 (Wang & Macrina, 1995), staphylococcal plasmid pGO1 (Climo *et al.*, 1996) and *A. tumefaciens* plasmid pTiC58 (Cook & Farrand, 1992). The inverted repeat sequence which is commonly found in the upstream region of the *oriT* sequence was also present in pKJ50 (IRVI). These results suggest the possibility of gene transfer mechanisms via bacterial conjugation in the genus *Bifidobacterium*. However, no

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pKJ50  M-----RDERTGEAFNGFGR-RSASSMYARCCPRA-----RPA
pIP501  MTI-----AKRENGKRSLIAMASYRSGEKLYSELYEKTNLNHRVTKPE
RSF1010 MAIYHLTAKTGRSRGGQSARAKADYIQREGKYARDMDEVL-HAESGHMPE
pSC101  MASYHLSVKTGGKGG---SASPADYIAREGKYAREKDSLEHKESGNMPA
      *                               *                               *

pKJ50  STSTRSVCST-----PSRWPR-NVPTRGPR---RRSWSFPCPASSTPAN
pIP501  AFILKPDYVPNEFLDRQTLWNKMELAEKSPNAQLCREVNVALPIELNNSD
RSF1010 FV-ERP----ADY-----WDAADLYERA-NGRLFKEVEFALPVELTLDQ
pSC101  WAAHKP----SEF-----WKAADTSERA-NGCTYREIEIALPRELKPQE
      ..                               *                               *

                                     xxPHuHuuuxxx
pKJ50  AFRALDFISWNITANGYACTYAIHTDK----DGRNPHAHILVANR---R
pIP501  QRMLIEDFVKDNFVNEGMIADVAIHRD----DENNPHAHIMLTMR---E
RSF1010 QKALASEFAQHLTGAERLPYTLAIHA----GGGNPHCHLMI SERINDG
pSC101  RLELVRDFVQQEIG-DRHAYQFAIHNPKAAIAGGEPHAIIMP SERINDG
      *                               ***                               ** * . . *

pKJ50  IDPKTGRWAAKSRSEFALDANGRI-----PVIDPDTGRQKIGARNRKVW
pIP501  VDSE-GNILNKSRIIPKLDENGNQIFNEKGQRVTYSIKTNDWGRKSLVSEIRKDW
RSF1010 IERPAAQWFKRYNGKTP-----BKGGAKTEALKPKA-----WLEQTREAW
pSC101  IHRDPEQYFKRANTKEP-----DAVAQKRHVSGKHRPNAKNTLLPRGRR-W
      ..                               *                               *

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Fig. 4. Alignment of various Mob proteins. The aligned motifs are extracted from complete alignments generated by program CLUSTAL v as described. Amino acid residues conserved in all the aligned sequences are marked as *. The highly conserved motif is indicated in bold type (u, hydrophobic residue, x, non-consensus residue). Data from: this study, pKJ50; Wang & Macrina (1995), pIP501; Frey *et al.* (1992), RSF1010; Drolet *et al.* (1990), pSC101.

evidence supporting this has been reported to our knowledge.

ORFII coding region

Hydropathy plot analysis suggests that ORFII is a transmembrane protein with two transmembrane amino acid segments (78–98 and 197–216). The first segment is located within a helical structure (65–110) as shown by the PROSIS program. In general, segments containing about 20–30 aa with a high degree of hydrophobicity are considered to be long enough to span a membrane as an α -helix (Eisenberg, 1984). The amino acid sequence homology search of the 171–214 segment showed 38% identity with transferrin-binding protein 2 of *Haemophilus influenzae* (Loosmore *et al.*, 1996) and the 159–204 segment showed 34% identity with the multi-drug resistance protein of *Candida albicans* (Prasad *et al.*, 1995). This suggests that ORFII may be a receptor membrane protein; however, its exact function requires elucidation.

Secondary structure

There are six direct repeats, eight inverted repeats and four stem-loop structures in the pKJ50 plasmid DNA sequence. DRI is 68 bp long and is followed directly by stem-loop III (SLIII). This sequence may play an important role in the control of plasmid replication by providing a target site for translation and transcription factors. DRV and DRVI display an interesting structure

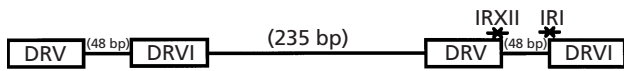


Fig. 5. Direct repeats with identical spacer length. DRV (4589–4612, 4915–4938) and DRVI (27–45, 4661–4679) display an interesting structure with spacers of identical length (48 bp) but different nucleotide sequence between each DRV/DRVI pair.

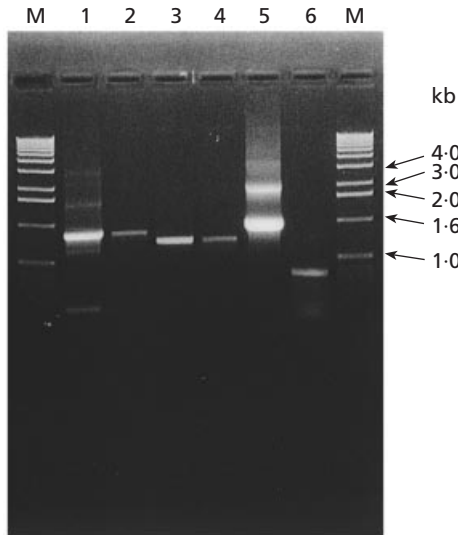


Fig. 6. Confirmation of expression of each ORF of pKJ50 by RT-PCR and PCR. Lanes: 1, PCR product with pKJ50 plasmid DNA template and primers MS005 and MS006; 2, RT-PCR product with total RNA from *B. longum* KJ and primers MS005 and MS006; 3, PCR product with pKJ50 plasmid DNA template and primers MS001 and MS002; 4, RT-PCR product with total RNA from *B. longum* KJ and primers MS001 and MS002; 5, PCR product with pKJ50 plasmid DNA template and primers MS003 and MS004; 6, RT-PCR product with total RNA from *B. longum* KJ and primers MS003 and MS004; M, molecular mass markers.

(Fig. 5) with identical spacers (48 bp) between the left- and right-hand DRV/DRVI pairs. Moreover, the right-hand DRV/DRVI pair is flanked with inverted repeats which may differentiate the two sets of direct repeats. However, the significance of these structures remains to be elucidated.

Confirmation of expression of ORFI and ORFII

Sequence analysis of pKJ50 suggests that this plasmid has three ORFs as described above. This was confirmed by RT-PCR and a coupled *in vitro* transcription/translation reaction using several DNA templates containing pKJ50 fragments and ^{35}S -methionine as chase material. RT-PCR was used to detect the expected mRNA that might be transcribed from each ORF. By using three primer sets specific for the upstream and downstream sequences of each ORF, PCR products of the expected size could be detected for ORFI and ORFII (Fig. 6). The size of each RT-PCR product was identical to that of the PCR product produced by using the same

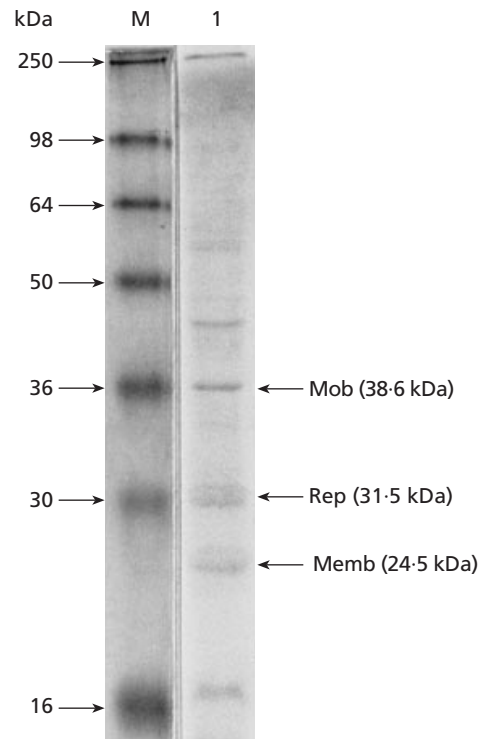


Fig. 7. Coupled *in vitro* transcription/translation of each ORF on pKJ50 (lane 1). Plasmid pKJ50 was expressed in the *E. coli* T7 S30 Extract System (Promega). The proteins were expressed as described in Methods using ^{35}S -methionine as chaser, resolved by SDS-PAGE (15% acrylamide) and visualized by autoradiography. Mob, putative mobilization protein; Rep, putative replication protein; Memb, putative membrane protein. Lane M, molecular mass markers.

primer sets and pKJ50 plasmid DNA. However, ORFIII, encoding the putative Mob protein, showed no RT-PCR product, although the PCR reaction with pKJ50 template produced a product of the expected size. This might suggest that ORFIII of pKJ50 is not usually expressed. When pKJ50 was used as template DNA, an *in vitro* translation reaction produced three major protein bands positioned at 31, 24 and 38 kDa, corresponding to each ORF (Fig. 7).

Shuttle vector construction

E. coli-*B. animalis* shuttle vectors pBKJ50F and pBKJ50R were constructed as described in Methods and Fig. 1. They were designed not to disturb the putative Rep protein. They were transformed into *B. animalis* MB209 with an efficiency of 2.0×10^2 c.f.u. ($\mu\text{g DNA}$) $^{-1}$ by electroporation at 10 kV cm^{-1} , 200Ω and $40 \mu\text{F}$, and selected using $3 \mu\text{g}$ chloramphenicol ml^{-1} . The integrity of pBKJ50F was confirmed by PCR using the same sets of primers as above and comparing Southern hybridization patterns of plasmid DNA extracted from *B. animalis* transformants with those of pBKJ50. pBKJ50 and the plasmid isolated from the transformants showed the same PCR products and Southern hybridization

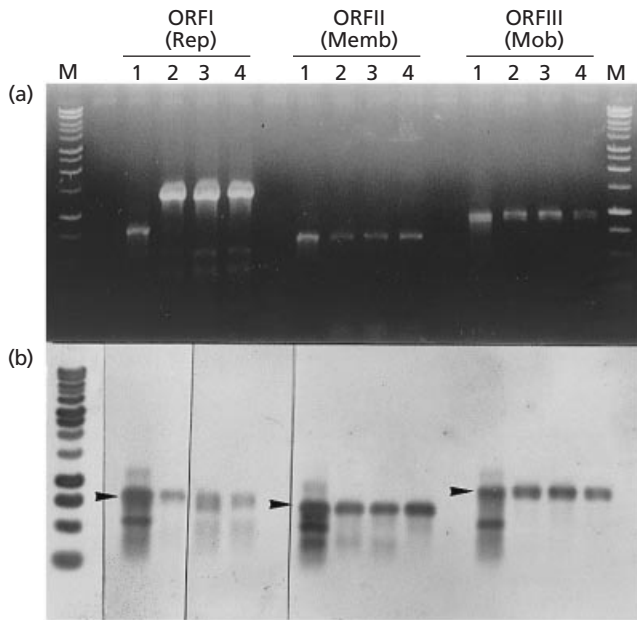


Fig. 8. Confirmation of transformation of *B. animalis* MB209 with pBKJ50 by PCR and Southern hybridization. The same sets of primers were used as described in the legend to Fig. 6. (a) PCR products amplified from each ORF separated by electrophoresis. (b) PCR products were hybridized with pBKJ50 as probe and the specific products of each ORF are indicated with arrows. Lanes 1 and lanes 2–4 show PCR products and Southern hybridization patterns, respectively, of pBKJ50 and plasmids extracted from *B. animalis* MB209 transformants.

patterns, revealing that the plasmid was successfully transformed (Fig. 8a, b).

This study has shown that Rep and Mob proteins of the strictly anaerobic bacterium *B. longum* KJ are highly homologous with those of diverse and phylogenetically distant micro-organisms, and the expression of the putative Rep and membrane proteins has been investigated. It is possible that the rolling-circle mechanism of plasmid replication and mobilization is employed by *B. longum* KJ. However, the presence of a different Rep protein in pMB1 of *B. longum* B2577 and pAP1 of *B. asteroides* suggests that at least two different modes of plasmid replication are possible in various *Bifidobacterium* strains. In addition, an *E. coli*–*B. animalis* shuttle vector was constructed and successfully transformed into *B. animalis* MB209. This shuttle vector should be a good candidate for the construction of improved cloning vectors, such as food-grade cloning and expression vectors, which can be used in *Bifidobacterium* species.

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