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Characterization of the plasmid pMB1 from *Bifidobacterium longum* and its use for shuttle vector construction

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SUMMARY

The nucleotide sequence of the 1847-bp *Bifidobacterium longum* B2577 cryptic plasmid pMB1 was determined. The plasmid had a G+C content of 62.0%, and contained two open reading frames, *orf*1 and *orf*2, likely arranged in an operon. The proteins encoded by *orf*1 and *orf*2 show the highest degree of similarity with similarly arranged peptide sequences translated from *Corynebacterium glutamicum* pXZ10142 and *Mycobacterium fortuitum* pAL5000 plasmids. Recombinant plasmids containing the pMB1 replicon were able to replicate in *Bifidobacterium animalis* MB209. The successful transformation of this strain with pMB1-based plasmids facilitated characterization of this replicon, results of which showed that both *orf*1 and *orf*2 are necessary for plasmid replication. A family of new *Escherichia coli-B. animalis* shuttle plasmids, based on the pMB1 replicon and expressing a *cat* and an *ery* gene, was constructed.

Key-words: Plasmid, *Bifidobacterium longum*; Nucleotide sequence, Shuttle vector.

INTRODUCTION

Bifidobacteria constitute a major part of the normal intestinal microflora in humans and other animals and play a fundamental role in the health of the host. The microflora composition is rather dynamic and *Bifidobacterium* spp. are fundamental to the maintenance of a correct balance. They compete with intestinal putrefactive bacteria, such as enterobacteria (Ibrahim and Bezkorovainy, 1993) and clostridia (Bezirtzoglou and Romond, 1990), lowering the pH of the gut. They produce mainly acetate and lactate from carbohydrate catabolism, which inhibit pathogenic bacteria.

Another mechanism of resistance to infections which is due to bifidobacteria is the stimulation of immune response (Yashi and Ohwaki, 1991; Lee *et al.*, 1993), providing protection of the host against virus infections, such as murine rotavirus diseases (Duffy *et al.*, 1994; Saavedra *et al.*, 1994). Furthermore, epidemiological and experimental studies have provided evidence that fermented milk containing *Bifidobacterium* spp. reduces the risk of certain types of cancer (Van't Veer *et al.*, 1989; Reddy and Rivenson, 1993). Possible explanations include enhanced immunostimulation and the lowering of the levels of undesirable enzymes (β-glucuronidase, nitrore-

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ductase, azoreductase, β-glucosidase) (Daly, 1991; Kurmann and Rasic, 1991), ammonia (Deguchi *et al.*, 1993) and N-nitroso compounds that are potential carcinogens.

Because of the wide probiotic activity of these microorganisms, *Bifidobacterium* spp. are largely used both in pharmaceutical and dairy preparations. The possibility of improving the characteristics of some strains by molecular biological techniques is very promising. In particular, these microorganisms are potential hosts for the construction of oral vaccines and for the cloning of genes encoding detoxifying activities, such as cholesterol oxidase and bile salt hydrolase.

Application of recombinant DNA technology to bifidobacteria has been delayed by past difficulties associated with the transformation of members of this genus. It was demonstrated only recently that plasmid DNA can be introduced in *B. longum* by electroporation (Missich *et al.*, 1994).

The presence of plasmids was demonstrated in strains of *B. breve*, *B. longum*, *B. asteroides*, *B. indicum* and *B. globosum*, all of which remain cryptic (Sgorbati *et al.*, 1982, 1986a, 1986b; Iwata and Morishita, 1989). Two cloning vectors, based on the *B. longum* B2577 plasmid pMB1, were introduced into *Escherichia coli* by electroporation, but could not be transferred to *Bifidobacterium* spp. or other Gram-positive hosts (Matteuzzi *et al.*, 1990). The vector pRM2, the first plasmid to be successfully transferred to *B. longum*, is based on the pMB1 replicon (Missich *et al.*, 1994). In this study, an analysis of the replication functions of pMB1 replicon was initiated by determining the complete nucleotide sequence of the plasmid and the minimal region required for replication. The successful transformation of *B. animalis* MB209 with pMB1-based vectors, and information obtained from the sequence of pMB1 facilitated the characterization of this replicon and the construction of new vectors able to replicate in this host.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

E. coli HB 101 (λ⁻ *recAB proA2 leu lacY1 galK2 xyl-5 mtl-1 ara-14* [F⁻ *hsdS20*] (r_{B-} m_{B-}) (Sambrook *et al.*, 1989) was used for general cloning purposes, while *E. coli* TG2 (Δ (*lac-pro*) *supE thi hsdM hsdR recA* [F' *traD36 proAB*⁺ *lacZΔM151*], from T.J. Gibson, was used for subcloning and ssDNA production for sequencing. *E. coli* was grown in Luria broth at 37°C.

B. longum B2577 (Matteuzzi *et al.*, 1990) contains the plasmid pMB1, studied in this paper; *B. animalis* MB209 (our collection) was the host for transformation. *Bifidobacterium* spp. were grown anaerobically at 37°C in MRS (Difco) containing 0.05% cysteine.

Antibiotics were added to the appropriate media at the following concentrations (μg/ml): for *E. coli*, ampicillin 100 and chloramphenicol 30, for *B. animalis* MB209, chloramphenicol and erythromycin 5 each.

All the plasmids used in this study are listed in table I.

Biochemicals and reagents

Restriction and modification enzymes, from Boehringer Mannheim, were used as suggested by the supplier. Reagent grade chemicals were obtained from Merck or Sigma.

DNA purification, manipulation and transformation

Plasmid DNA was isolated from *Bifidobacterium* spp. according to LeBlanc and Lee (1979). Minipreps and large scale *E. coli* plasmid purifications were described as in Sambrook *et al.* (1989). ssDNA preparations were obtained as detailed by Bankier and Barrel (1983), after coinfection with VCSM13 helper phage (Promega).

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bp	=	base pair.	*ori*	=	origin of DNA replication.
cat	=	chloramphenicol acetyltransferase gene.	*orf*	=	open reading frame.
ery	=	erythromycin resistance gene.	nt	=	nucleotide.
kb	=	kilobase.	RBS	=	ribosome-binding site.
kDa	=	kilodalton.	ss	=	single strand(ed).

Table I. Plasmids used in this study.

Plasmid	Description	Reference
pMB1	Cryptic plasmid isolated from *B. longum* B2577 (1.8-kb)	Sgorbati *et al.*, 1982
pBluescriptK+	Phagemid derived from pUC19 (2.9-kb)	Stratagene
pJH101	pBR322 derivative containing the *cat* gene from pC194 (5.4-kb)	Ferrari *et al.*, 1983
pEI2	pUC19 derivative containing the *ery* gene from pE194 (3.9-kb)	Posno *et al.*, 1991
pDG7	1.7-kb *Eco*RV fragment of pMB1 cloned in pJH101 *Eco*RV site	Matteuzzi *et al.*, 1990
pMR3	pMB1 linearized *Pvu*II and cloned in pJH101 *Eco*RV site	Matteuzzi *et al.*, 1990
pSS1	1.7-kb *Eco*RV fragment of pMB1 cloned in pBluescriptKS+ *Eco*RV site	This study
pSS3	1.7-kb *Eco*RV fragment of pMB1 cloned in pBluescriptKS+ *Eco*RV site (opposite orientation than pSS1)	This study
pMM1	pMB1 linearized *Pvu*II and cloned in pBluescriptKS+ *Eco*RV site	This study
pMM3	pMB1 linearized *Pvu*II and cloned in pBluescriptKS+ *Eco*RV site (opposite orientation than pMM1)	This study
pNC7	4.9-kb fragment *Nde*I-*Cla*I of pDG7 filled-in and self-ligated	This study
pDH7	1.2-kb fragment *Hae*II of pMB1 cloned in pJH101 *Eco*RV site	This study
pKG7	1.6-kb *Ksp*I-*Eco*RV fragment of pMB1 filled-in and cloned in pJH101 *Eco*RV site	This study
pDGE7	1.2-kb *Eco*RI-*Hin*dIII from pEI2 (*ery* gene) cloned in pDG7 digested *Eco*RI-*Hin*dIII	This study

Purification of DNA restriction fragments from agarose gel was accomplished by use of the Qiaex Kit (Qiagen Inc.). DNA manipulations (restriction enzyme cleavage, ligation and fill-in reactions with T4 DNA polymerase) were carried out under conditions specified by the manufacturer, or according to standard protocols (Sambrook *et al.*, 1989).

Plasmid constructs were introduced into *E. coli* HB101 by electroporation (Dower *et al.*, 1988). Transformation of *E. coli* TG2 cells by the $CaCl_2$ method was carried out essentially according to Sambrook *et al.* (1989). *B. animalis* MB209 was transformed by the protocol of Argnani *et al.* (1995). A gene pulse apparatus (Bio-Rad laboratories) equipped with a pulse controller unit was used for all electroporation experiments described in this study.

Strategy for cloning and sequencing pMB1

pMB1 was digested with *Eco*RV and cloned in pBluescriptIIKS+. The resulting plasmids pSS1 and pSS3 contained pMB1 in opposite orientations. A family of subclones, representing a series of nested deletions generated from pSS1 and pSS3, was obtained by using the Double-Stranded Nested Deletion Kit (Pharmacia). The nucleotide sequence of both strands was determined with the Sequenase Kit (USB). ssDNA of pSS1 and pSS3-derived subclones was obtained by coinfection with VCS-M13 helper phage.

To sequence the 99-bp fragment (nt 1-99) lost when the largest of the *Eco*RV restriction fragments of pMB1 was cloned, pMB1 was linearized by digestion with *Pvu*II, and cloned into *Eco*RV-digested pBluescriptIIKS+. The ssDNAs obtained with the recombinant plasmids containing the plasmid in opposite orientations (pMM1 and pMM3), were sequenced by using the primers 5' ATCGGCCTTCGGCACTCGGG and 5' GAAGAAGACCAGGATTTGCC, respectively.

The sequence data obtained were compiled and analysed on a VAX computer using the Genetic Computer Group Programs Package, Madison, WI, USA.

Vector constructions

pDH7. The 1.2-kb *Hae*II fragment of pMB1 (nt 195-1387) was gel-purified and cloned in pJH101, linearized by digestion with *Eco*RV. The plasmid derivative, with the pMB1 insert in the same orientation as in pDG7, was designated pDH7; it lacks *orf*2 of pMB1 and about 200 bp upstream from *orf*1.

pKG7. The 1.6-kb *Ksp*I-*Eco*RV (nt 198-1847) fragment of pMB1 was gel-purified, end-repaired with T4 DNA polymerase and dNTPs and cloned in pJH101, linearized by digestion with *Eco*RV. The plasmid derivative, with the same orientation of the inserted DNA as pDG7, was designated pKG7. It has the same pMB1 insert as pDG7 except for the deletion of 200 bp upstream from *orf*1.

pNC7. The 4.9-kb *Nde*I-*Cla*I fragment of pDG7 was gel-purified, end-repaired with T4 DNA polymerase and dNTPs and self-ligated. The plasmid obtained, pNC7, has the same pMB1 insert as pDG7, but it lacks the ampicillin resistance gene and the *E. coli* origin of replication.

pDGE7. The erythromycin resistance gene from pEI2, contained within an *Eco*RI-*Hin*dIII fragment (Posno *et al.*, 1991), was cloned into *Eco*RI/*Hin*dIII-digested pDG7.

RESULTS

pMB1 nucleotide sequence

The complete sequence of pMB1, shown in figure 1, provided a size for the plasmid of 1847 bp, with a calculated G+C content of 62%. A codon probability profile (fig. 2), based on the codon usage of the *B. longum* L-lactate dehydrogenase gene (*ldh*) (Minowa *et al.*, 1989), suggested the presence in pMB1 of at least two open reading frames (*orf*1 and *orf*2).

*orf*1 (nt 444-1361) occurred in frame 3 and had the strongest coding preference and fewest rare codons. It encoded a putative polypeptide (Orf1) of 36.8 kDa with a calculated isoelectric point of 7.57. Its start codon was not identified because the corresponding ribosome-binding site could not be located. The start codon in position 444 is only a reference codon.

A promoter search was conducted by comparison with *ldh* promoter sequences. There was a possible sequence for the promoter TTGACT-(19 bp)-TATATT, 192 bp upstream from the *ldh*-gene start codon, that is homologous to the consensus sequences for *E. coli* promoters (Rosenberg and Court, 1979). The *ldh* transcription start point was mapped by S1 nuclease and the putative sequence for the *ldh* promoter has been ascribed to GTAGCAA-(14 bp)-TATAGA, 51 bp upstream from the start codon. The sequence TTCAGC-(21 bp)-TATCTG, also similar to the *E. coli* consensus promoter sequence, was located at position 374 of pMB1, while the sequence GTAGCCA-(11)-TATTCG, highly homologous to the *ldh* promoter proposed by S1 mapping, was seen at position 451. It is of interest that both regions corresponding to the possible *ldh* promoters were present upstream from *orf*1, in the same order and spaced by almost the same number of residues. If the second promoter proposed for the *ldh*-gene were functional, it would be located downstream from the *orf*1 reference start codon.

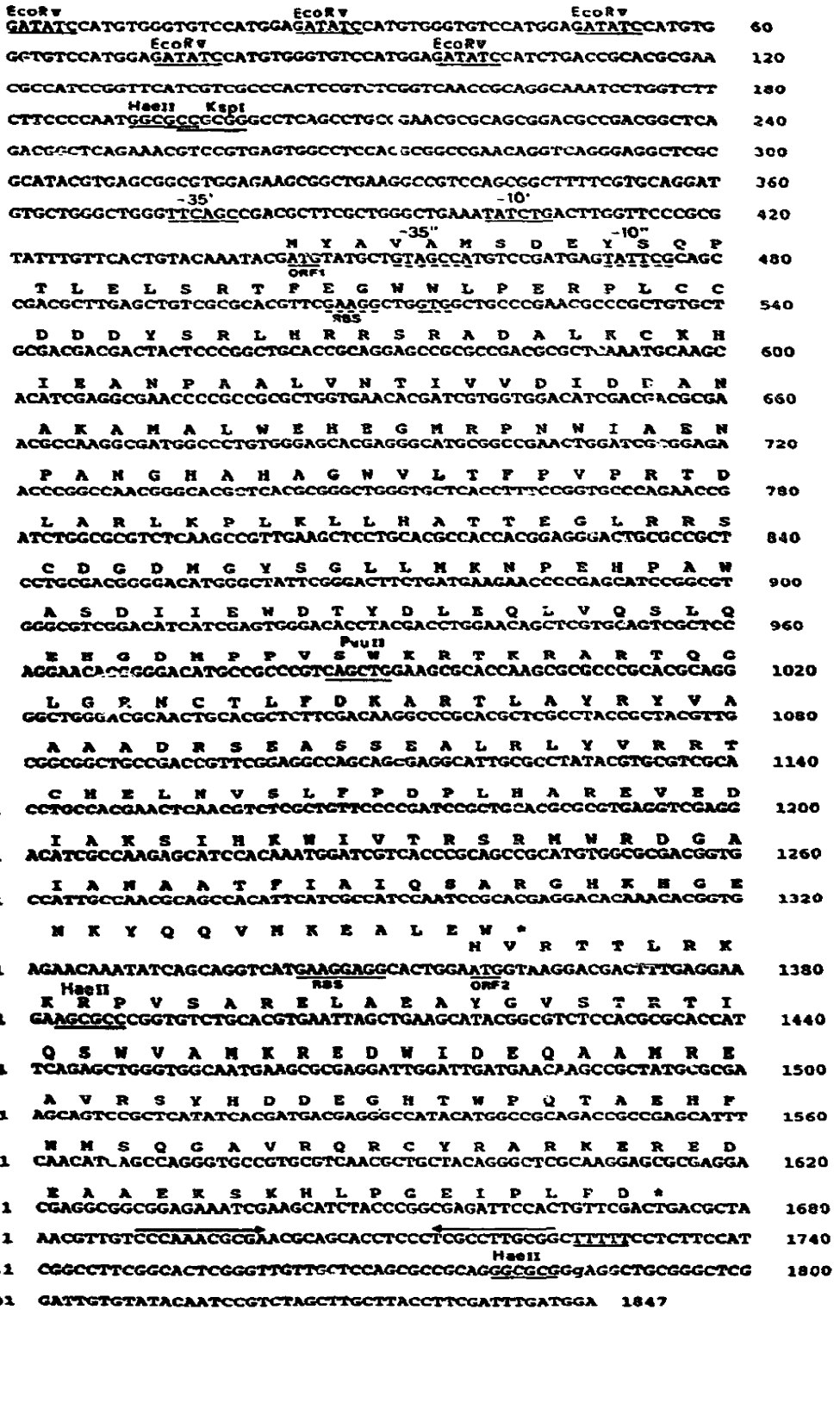
*orf*2 (nt 1358-1672) occurred in frame 2 and encoded a putative polypeptide of 12.6 kDa (Orf2), with an isoelectric point of 8.44. Its first codon ATG overlapped the *orf*1 stop codon TGG. A putative ribosome-binding site GAAGGAGG-(7 nt)-ATG was located 7 bp upstream from the start codon but no -35 and -10 promoter sequences similar to those of *ldh* could be identified. Most likely, the two genes are part of the same operon because the overlapping stop and start codons are an arrangement typical for many bacterial operons. Moreover, downstream from the stop codon of *orf*2, but not *orf*1, there is a strong potential rho-independent transcription terminator consisting of an inverted repeat sequence followed by a string of U's.

Similarities of Orf1 and Orf2 to proteins encoded by other plasmids

No information is available concerning genes encoded by *Bifidobacterium* spp. plasmids. To

Fig. 1. Complete nucleotide sequence of the *B. longum* plasmid pMB1 and deduced amino acid sequences for Orf1 and Orf2.

The start codons of *orf*1 and *orf*2 are underlined; the broken underlines show another possible start codon GCG (not in frame) for *orf*1, its putative RBS and the corrisponding promoter (–35" and –10"). The other hypothetical promoter is underlined (–35' and –10'). The stop codons are indicated by a single asterisk (*). A putative transcription terminator is present at nt 1689 to 1730, the inverted repeats of which are marked by arrows and the string of T's underlined. The restriction sites used for cloning have also been marked.


EcoRV
EcoRV
EcoRV
EcoRV
EcoRV
HaeII
KspI
-35'
-10'
-35"
-10"
ORF1
RBS
PvuII
HaeII
RBS
ORF2
HaeII

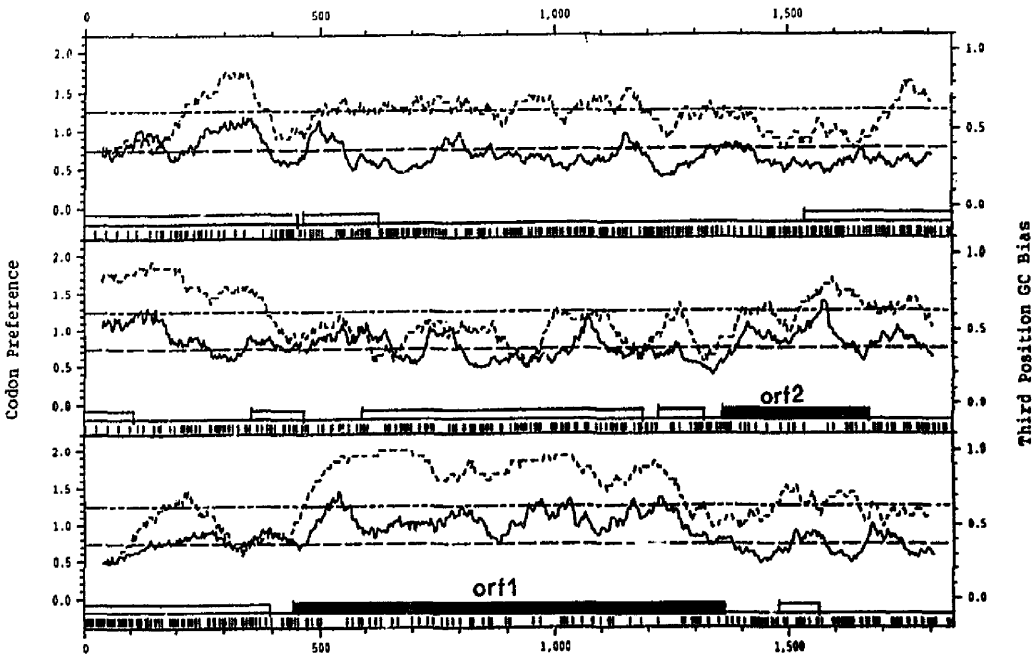


Fig. 2. Codon probability profile of the pMB1 sequence, based on codon preference and third position G+C bias.

The predicted *orf*1 and *orf*2 are indicated.

identify potential functions of proteins encoded by the two *orf*s described above, homology searches for the deduced amino acids sequences in GenBank, EMBL and SwissProt data bases were performed.

The proteins encoded by *orf*1 and *orf*2 exhibited similarity to peptide sequences translated from *Corynebacterium glutamicum* plasmid pXZ10142 (sequence unpublished, accession number X72691) and *Mycobacterium fortuitum* plasmid pAL5000 (Rauzier *et al.*, 1988; Labidi *et al.*, 1992). Orf1 was 61.5% similar and 46.0% identical to the corresponding pXZ10142 peptide, and 57.4% similar and 41.9% identical to that encoded by pAL5000. Orf2 exhibited very good similarity and identity (50.9% and 66.3%, respectively) with a pAL5000 deduced peptide, and significant, but less similarity and identity with the corresponding protein of pXZ10142 (36.6% and 15.5%, respectively). The alignment of Orf1 and Orf2 with the corresponding pAL5000 and pXZ10142 peptides is shown in figure 3. The pAL5000 and pXZ10142 *orf*1 stop codons overlapped the corresponding *orf*2 start codons, as also observed for pMB1.

Good homology was also observed between Orf1 and Orf2 of pMB1 and polypeptides encoded by the *Neisseria gonorrhoeae* plasmid pJD1 (Korch *et al.*, 1985). Orf1 was 50.8% similar and 31.4% identical to a deduced pJD1 peptide; Orf2 was 50.0% similar and 31.2% identical to a corresponding pJD1 peptide. Also in pJD1, the two genes have a tandem structure, with the stop codon of the first spaced by three nucleotides from the start codon of the second.

pMB1 Orf1 also was similar to ColE-type plasmid Rep proteins (ColE2-P9, ColE3-CA38, ColE4-CT9, ColE5-099, ColE2-GEI602, ColE2-

A

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          1                                                                  50
B1                                MYAVA MSDEYSQFTL ELSRTFEGWW LPERPLCCDD
M1  VGWPLSEISD LELVSDHTPV INRGLLPSVS HVADEFEQLW LPYWPLASDD
C1                                      MRPST RPEGRFEQLW LPLWPLASDN

          51                                                                 100
B1  DYSRLHRRSR ADALKCKHIE ANPAALVNTI VVDIDDANAK AMALWEHEGM
M1  LLEGIYRQSR ASALGRRYIE ANPTALANLL VVDVDHPDAA LRALSARGSH
C1  LRDGIYRTSR DKALDKRYIE TNPDAISNLL VVDIDKQNAL LRSMWDRDGW

          101                                                                150
B1  R.PNWIAENP A.NGHAHAGW VLTFPVPRTD LARLKPLKLL HATTEGLRRS
M1  PLPNAIVGNR A.NGHAHAVW ALNAPVPRTE YARRKPLAYM AACAEGLRRA
C1  R.PNAVVENP XLNGHAHAVW ALAEPFTRTE YARRKHXAYA AAVTEGLRRS

          151                                                                200
B1  CDGDMGYSGL LMKNPEHPAW ASDIIEWDTY DLEQLVQSLQ EHGDMPPVSW
M1  VDGDRSYSGL MTKNPGHIAW ETEWLHSDLY TLSHIE..AE LGANMPPPRW
C1  VDGDKGYSGL ITKNPEHTAW DSHWVTDKLY TLDELRFWLE ETGFMPPESW

          201                                                                250
B1  KR..TKRART QGLGRNCTLP DKARTLAYRY VAAAADRSEA SSEALRLYVR
M1  RQQTTYKAAP TPLGRNCALF DSVRLWAYRP ALMRIYLPTR NVDGLGRAIY
C1  KK..TRRKSP IGLGRNCALF ESARSWAYRE ..IRHFFG.. DPDGLGRSIQ

          251                                                                300
B1  RTCHELNVSL .....FPDPL HAREVEDIAK SINKWIVTRS RMWRDGAIAN
M1  AECHARNAEF PCNDVCPGPL PDSEVRAIAN SIWRWITTKS RIWADGIVVY
C1  ATAQALNQEL .....FSEPL PVAEVDQIAR SIHRWIITKS RMWTDGPAVY

          301                                                    339
B1  AATFIAIQSA RGHKHGENKY QQVMKEALEW .........
M1  EATLSARQSA ISRKGAAART AASTVARRAK SASAMEALL
C1  EATFTTIQAT RGKRSAEHRW GTTNAERIER FIND.....
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B

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          1                                                                  50
B2  ..........  ...MVRTTLR KKRPVSAREL AEAYGVSTRT IQSWVAMKRE
M2  MSDGYSDGYS  DGYNRQPTVR KKRRVTAAEG ARITGLSERH VVRLVAQERS
C2  ..........  ...MTKRTRI PRNGKTIREV AEGTGLSTAT IERWTSAPRE

          51                                                                 100
B2  DWIDEQAAMR EAVRSYHDDE GHTWPQTAEH FNMSQGAVRQ RCYRARKER.
M2  EWLAEQAARR ERIRAYHDDE GHSWPQTAKH FGLHLDTVKR LGYRARKERA
C2  DYLAQANEKR VRVQELR.AK GLSMRAIAAE IGCSVGLVHR YV....KEVE

          101        120
B2  .EDEAAEKSK HLPGEIPLFD
M2  AEQEAAQKAH NEADNPPLF.
C2  EKKTA..... ..........
```

Fig. 3. Comparison of Orf1 (B1) (A) and Orf2 (B2) (B) with the corresponding amino acid sequences deduced from pAL5000 *M. fortuitum* and pXZ10142 *C. glutamicum* plasmids. (M1 and C1 (A); M2 and C2 (B)).

Bold characters indicate the residues conserved among at least two sequences.

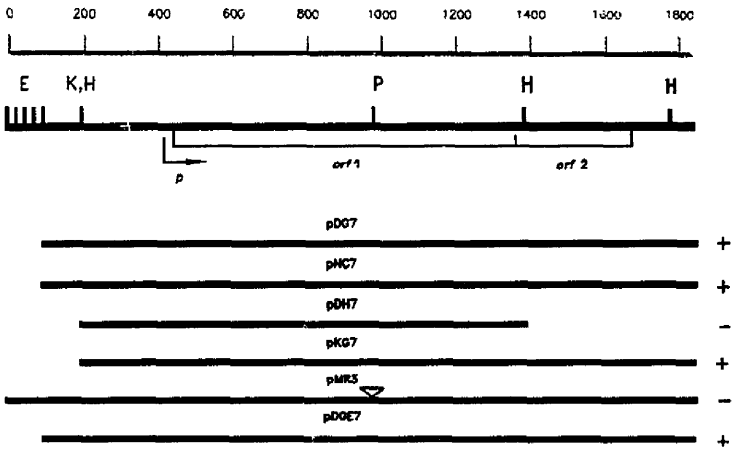

Fig. 4. Restriction map and scale (nt) of the plasmid pMB1 (E, *Eco*RV; K, *Ksp*I; H, *Hae*II, P, *Pvu*II) *orf*1, *orf*2 and the putative promoter are shown.

Open bars represent the insertion fragment from plasmid pMB1 present in vectors; + or – indicate that the vector replicates in *B. animalis* MB208 or that it does not.

K317, ColE2-CA42, ColE7-K317, ColE9-J, ColE6-CT14, ColE8-J), that are *trans*-acting factors required for autonomous replication (Yasueda *et al.*, 1989). This family of highly homologous proteins exhibited 51.3% similarity and 29.7% identity with Orf1.

E. coli-Bifidobacterium shuttle vectors

The successful transformation of *B. animalis* strain MB209 (3.7×10^3 transformants per µg DNA), (Argnani *et al.*, 1995), developed using the vector pDG7 (Matteuzzi *et al.*, 1990) allowed the construction of new *E. coli-B. animalis* shuttle vectors which were used to characterize elements of pMB1 essential for replication.

Most of the new plasmids contained pJH101 (Ferrari *et al.*, 1983), which is pBR322 with the *cat* gene from pC194 cloned into the *Pvu*II site. pJH101 replicates in *E. coli*, but not in Gram-positive hosts. The pMB1 fragments contained in these vectors are presented in figure 4.

pDG7 contained the large *Eco*RV fragment of pMB1, and lacked only the 95 bp included in the four small contiguous *Eco*RV fragments, cloned into *Eco*RV linearized pJH101. Thus, the region included between nt 0 and 105 (see fig. 1), containing the repeated sequences and having a strong secondary structure, was not necessary for plasmid replication.

Neither pMR3- (Matteuzzi *et al.*, 1990) nor pDH7-transformed *B. animalis* MB209. pMR3 contained *Pvu*II linearized pMB1 in which *orf*1 was interrupted by the insertion of pJH101, while pDH7 lacked *orf*2. These data suggest that *orf*2 was involved in plasmid replication.

Compared to pDG7, pDH7 lacked *orf*2 and a 95-bp fragment included between *Eco*RV (nt 99 of pMB1) and *Hae*II (nt 195). To demonstrate that pDH7 did not replicate because of the absence of *orf*2, and not because of the lack of the 95-bp fragment, a pDG7 derivative lacking only the fragment included between nt 0 and 198 was constructed. The plasmid obtained, designated pKG7, transformed *B. animalis* MB209, suggesting that the pMB1 region included between nt 0 and 198 is not necessary for replication, and that the unsuccessful transformation with pDH7 was due to the absence of *orf*2.

Plasmid pNC7 was the smallest of the family (4.9-kb). It contained the same pMB1 fragment as pDG7, but it lacked the β-lactamase resistance gene and the *E. coli* origin of replication. It transformed *B. animalis* MB209 and replicated stably. pNC7 was used to transform *E. coli* by electroporation to determine whether pMB1 could replicate in this host. Only a few transformants per μg of DNA were obtained after 2 days of incubation. Plasmids obtained from these transformants had electrophoretic patterns and restriction maps different from the original pNC7. Therefore, pNC7 underwent rearrangements and deletions in *E. coli*.

In the perspective of direct cloning in *Bifidobacterium* spp. and selection by insertional inactivation of an antibiotic marker, the erythromycin resistance gene from *Staphylococcus aureus* was cloned in pDG7, which contains the *cat* gene. The recombinant plasmid pDGE7 replicated well in *B. animalis*, and both the antibiotic resistance genes were expressed.

Except for pNC7, all plasmids described replicated stably in *E. coli* and did not rearrange in this host. Those that transformed *B. animalis* MB209 were structurally stable in this host.

Nucleotide sequence accession number

The pMB1 sequence has been deposited in the EMBL Nucleotide Sequence Database under accession number X84655.

DISCUSSION

The complete nucleotide base sequence of pMB1 was determined and the successful transformation of *B. animalis* MB209 with the pMB1-based plasmid pDG7 enabled characterization of this replicon. pMB1 is the first *Bifidobacterium* plasmid to be sequenced. Analysis of this sequence revealed a high degree of homology with *C. glutamicum* and *M. fortuitum* plasmids. *Corynebacterium* spp., *Mycobacterium* spp., and *Bifidobacterium* spp. are Gram-positive bacteria belonging to the high-G+C subdivision, that is, greater than 55% G+C (Woese, 1987). The anaerobic species of bifidobacteria and propionibacteria occupy the deepest branchings of this subdivision. However, this subgroup as a whole is not particularly deep, which may explain the high degree of similarity of *B. longum* pMB1 sequences with those of plasmids from *C. glutamicum* and *M. fortuitum*. Close genetic relationships between mycobacteria and corynebacteria also were supported by the use of a *C. glutamicum* replicon for the construction of *Mycobacterium-E. coli* shuttle vectors (Radford and Hodgson, 1991).

The two highly homologous tandem genes present in *B. longum*, *M. fortuitum*, *C. glutamicum* and *N. gonorrhoeae* plasmids suggest the possibility of a two-gene operon shared by a wide family of plasmids belonging not only to phylogenetically related bacteria, but also to unrelated species such as the Gram-negative *N. gonorrhoeae*. The arrangement of this putative operon was suggested by the location in pMB1 of putative promoter sequences upstream from *orf*1, and of the rho-independent termination signal downstream from *orf*2.

No information is available concerning the mechanism of replication of these plasmids, but the significant similarity between Orf1 and ColE-type Rep proteins is highly suggestive of the involvement of the *orf*1 product in plasmid replication. For ColE-2, which has been intensely studied (Yasueda *et al.*, 1989, 1994; Takechi *et al.*, 1994), it has been demonstrated that the Rep protein, similar to Orf1, is the *trans*-acting product responsible for initiation of replication. By analogy, we suggest that Orf1 may be the pMB1 Rep protein. The unsuccessful replication in *B. animalis* MB209 of vector pMR3, which had *orf*1 interrupted, could be due either to the absence of the *orf*1 product or to a polar effect on *orf*2, or both. Downstream from the ColE2 *rep*-gene, there is a second open reading frame that is not functional (*orf*2); here is located the ColE2 origin of replication, consisting of two direct repeat sequences. The pMB1 origin of replication was not established. The region encompassed by nucleotides 1-105 (see fig. 1) is very rich in direct and inverted repeat sequences, but it was not present in plasmids pDG7 and pKG7, both of which replicated in *B. animalis*. To check whether

the putative pMB1 Rep protein, Orf1, can use the pJH101 origin of replication, pNC7 was constructed. This plasmid, lacking *E. coli ori*, replicated stably in *B. animalis* MB209, while it transformed *E. coli* with very low efficiency, where it underwent rearrangements and deletions (data not shown). Therefore, the pMB1 origin of replication is not located in the first 105 nucleotides, and an *E. coli ori* is not necessary for pMB1-based plasmids to replicate in *B. animalis* MB209.

Acknowledgements

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Le plasmide pMB1 de *Bifidobacterium longum* est caractérisé et utilisé pour la construction d'un vecteur navette

Nous avons déterminé la séquence nucléotidique du plasmide cryptique pMB1 de 1847 bp de *Bifidobacterium longum* B32577. Le plasmide a un contenu G+C de 62%, et il renferme deux *orf*s (open reading frames), *orf*1 et 2, probablement agencés dans un opéron. Le plus haut degré d'homologie des protéines codées par *orf*1 et 2 est observé avec les séquences des peptides obtenues par translation des plasmides pXZ10142 de *Corynebacterium glutamicum* et pAL5000 de *Mycobacterium fortuitum*. Les plasmides recombinants contenant le réplicon pMB1 sont capables de se répliquer chez *Bifidobacterium animalis* MB209. La transformation réussie de cette souche avec ces plasmides a facilité la caractérisation du réplicon, ces résultats montrant que les *orf*1 et 2 sont nécessaires à la réplication des plasmides. Nous avons construit une famille de nouveaux plasmides navettes *Escherichia coli-B. animalis* basés sur le réplicon pMB1 et exprimant un gène *cat* et un gène *ery*.

Mots-clés: Plasmide, *Bifidobacterium longum*; Séquence nucléotidique, Vecteur navette.

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