Sequence analysis of plasmid pSP02 from *Bifidobacterium longum* M62 and construction of pSP02-derived cloning vectors

Pablo Álvarez-Martín[1,2‡], Joanna Życka-Krzesińska[1,2‡], Jacek Bardowski[2], and Baltasar Mayo[1*]

Department of Microbiology and Biochemistry, Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Carretera de Infiesto, s/n, 33300-Villaviciosa, Asturias, Spain[1], and Institute of Biochemistry and Biophysics of Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warszawa, Poland[2]

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[‡]These authors contributed equally to this work.

[*]Corresponding author: Baltasar Mayo, Departamento de Microbiología y Bioquímica de Productos Lácteos, Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain

Tel: +34 985 89 21 31

Fax: +34 985 89 22 33

e-mail: baltasar.mayo@ipla.csic.es

Abstract

Replicons from bifidobacteria species are required for the construction of general- and special-purpose vectors that would allow the undertaking of molecular studies of these bacteria. In this work, pSP02, a cryptic plasmid from *Bifidobacterium longum* M62, was cloned, sequenced and characterized. pSP02 was found to consist of 4896 bp with four ORFs coding for proteins over 50 amino acids long. Among the deduced protein sequences only a replicase (RepA) and a mobilization-like protein (MobA) showed known functional domains. Similar to previously described bifidobacterial plasmids, the organization of the putative *ori* region of pSP02 resembles that of the theta-replicating plasmids of Gram-positives. In spite of this, hybridization experiments detected single stranded (ss)-DNA as an intermediate product in the pSP02 replication, demonstrating it follows the rolling-circle (RC) replication mode. The *ori* region of pSP02 was used to construct a series of first generation cloning vectors able to replicate in many bifidobacterial species. Real time quantitative PCR established the copy number of pSP02 and its derived vectors to be around 12 copies per chromosome equivalent. pSP02-derivatives showed full segregational and structural stability even in the absence of antibiotic selection.

Introduction

Bifidobacterium species are among the dominant microbial populations of the gastrointestinal tract (GIT) of humans and other mammals, where they are considered to be responsible for a vast array of effects beneficial to health, including the production of short chain fatty acids, the exclusion of intestinal pathogens, and the modulation of immune function (Russell et al., 2011). These beneficial activities have led to the

extensive use of selected bifidobacterial strains as probiotics. However, the basic mechanisms by which bifidobacteria contribute to well-being remain poorly understood. This knowledge is extremely important for the long-term exploitation of bifidobacterial probiotics as promoters of health (Russell et al., 2011; Siciliano and Mazzeo, 2012).

The molecular study of bifidobacterial properties has traditionally been hampered by a lack of genetic tools (Ventura et al., 2004). Bifidobacteria have recently been envisioned as promising systems for the delivery of therapeutic agents such as antigens (for live vaccine development) and tumor-suppressing substances (Fujimori, 2006; Xu et al., 2007), but for this, stable and robust expression vectors are also needed. The lack of molecular tools further impedes the full exploitation of the genetic data provided by the recent genome sequencing of many bifidobacterial strains (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Since bacteriophages infecting *Bifidobacterium* species have never been characterized, and only a few prophage-like element have been identified in the bifidobacterial genomes (Ventura et al., 2005), plasmids would seem to be the only feasible type of replicon that can be used to construct these needed vectors.

To date over 40 plasmid sequences have been reported to the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore), and a few plasmid replicons have been studied in detail (O'Riordan and Fitzgerald, 1999; Park et al., 1999; Corneau et al, 2004; Lee and O'Sullivan, 2006; Park et al., 2008). Some of these have already been used to construct general cloning and expression vectors (Matsumara et al., 1997; Yazawa et al., 2001; Klijn et al., 2006; Rhim et al., 2006; Takata et al., 2006; Cronin et al., 2007; Álvarez-Martín et al., 2008), and some special-purpose vectors, such as bioluminescence reporter systems (Cronin et al., 2008; Guglielmetti et al., 2008).

However, more plasmids are still needed to develop compatible vector systems and construct food grade vectors and gene integration, disruption and replacement cassettes.

This work reports the molecular analysis of pSP02, a cryptic plasmid from *Bifidobacterium longum* M62. This replicon was used to construct first generation cloning vectors able to replicate stably in different bifidobacterial species and strains, including some used as commercial probiotics.

Material and Methods

Bacterial strains, cloning vectors and growth conditions

Table 1 shows the bacterial strains and vectors used in this study. Bifidobacteria strains were grown in anaerobiosis at 37ºC in MRS broth (Merck, Darmstad, Germany) or RCM broth (Merck) supplemented with 0.25% (w/v) L-cysteine (Sigma-Aldrich, St. Louis, MO, USA). *Escherichia coli* was grown with shaking at 37ºC in Luria Bertani (LB) broth (Sambrook and Russell, 2001). When required, antibiotics (all from Sigma-Aldrich) were added at the following concentrations: ampicillin 100 µg ml^{-1} and tetracycline 5 µg ml^{-1} for *E. coli* and erythromycin 5 µg ml^{-1} and tetracycline 5 µg ml^{-1} for bifidobacteria.

Isolation of plasmid DNA

Plasmid DNA from bifidobacteria was isolated using the method of O'Sullivan and Klaenhamer (1993) with the modification that pellets were suspended in TSE buffer (sucrose 25%, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and incubated with lysozyme (30 mg/ml) at 37ºC for 30 min. Plasmid DNA from *E. coli* was isolated using the

commercial QIAprep® Spin Miniprep kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's recommendations. Plasmids were analyzed by electrophoresis in TAE (40 mM Tris-HCl, 40 mM acetic acid, and 1 mM EDTA, pH 8.0), visualized under UV light and photographed.

Molecular DNA techniques

The general procedures followed for DNA manipulation were essentially those described by Sambrook and Russell (2001). Restriction endonucleases (Takara, Otsu, Shiga, Japan), Taq DNA polymerase (Ampliqon A/S, SkovlundeDenmark), the Klenow fragment of *E. coli* polymerase I and nuclease S1 (Roche Applied Sciences, Basel, Switzerland), and T_4 DNA ligase (Invitrogen, Carlsbad, CA; USA) were used according to the suppliers' instructions.

Cloning vectors and constructs were introduced into *E. coli* by electrotransformation (electroporation) using a Gene-Pulser Apparatus (Bio-Rad Laboratories, Richmond, CA, USA) according to Sambrook and Russell (2001). Electroporation of *Bifidobacterium* cells was performed under the following conditions: 25 μF, 200 Ω and 10 kV (Álvarez-Martín et al., 2007). Transformants were recovered on selective RCM agar plates incubated for 2-3 days at 37ºC under anaerobic conditions.

Sequencing and DNA sequence analysis

The complete pSP02 sequence was obtained by sequencing cloned plasmid *Bam*HI fragments into pUC18. In addition, the sequence of some segments was further verified by PCR amplification and sequencing of the amplicons. DNA and deduced protein sequences were examined for homology against non-redundant DNA and protein databases using the on-line BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNA hybridization

Plasmid DNA was transferred to Hybond-N nylon membranes (GE Health Care Bio-Sciences, Little Chalfont, UK) after electrophoresis and hybridized, using as a probe a digoxigenin-labeled internal segment of the *repA* gene obtained by PCR, under high-stringency standard conditions (hybridization at 68ºC and two final washing steps in 0.5 x SSC, 0.1% SDS at 68ºC for 15 min) (Sambrook and Russell, 2001).

For the detection of ssDNA, whole-cell lysates from *B. pseudocatenulatum* M115 harboring pSP1 were obtained and purified as described by Leenhouts et al. (1991). Unmodified DNA and DNA treated with nuclease S1 were then electrophoresed and transferred to a hybridization membrane under non-denaturing conditions. ssDNA was revealed by hybridization using the same *repA*-derived probe and labeling conditions as above.

Determination of the relative plasmid copy number

The relative copy number of pSP02 and its derived vectors was assessed by quantitative real-time PCR (qPCR) in a Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using Power SYBER® Green PCR Master Mix (Applied Biosystems), and employing the PCR conditions reported by Álvarez-Martín et al. (2008). Amplification of a segment of 139 bp was performed using two primers - CopyF and CopyR (Table 1)- based on the pSP02 *repA* sequence. A 148 bp segment of the single-copy Tu elongation factor gene of *B. longum* NCC2705 (*tuf*; GeneID: 1022637) was used as a reference. The relative copy number was calculated from triplicate experiments using the formula $N_{relative} = (1+E)^{-\Delta CT}$ (Lee et al., 2006), where E is

147 the amplification efficiency of the target and reference genes, and ΔCT the difference
148 between the threshold cycle number (CT) of the *tuf* reaction and that of *repA*.
149
150 **Segregation and structural stability of vectors**
151 The stability of the constructs was assayed in *B. pseudocatenulatum* M115 by
152 growing the cells in non-selective RCM-cysteine medium for approximately 100
153 generations and plating daily onto antibiotic-free agar plates. Plasmid segregation was
154 monitored by transference of the colonies to plates with and without antibiotics. Finally,
155 structural stability was monitored in representative resistant and susceptible colonies by
156 plasmid isolation and restriction analysis.
157
158 **Nucleotide sequence accession number**
159 The nucleotide and deduced amino acid sequences of pSP02 are available under
160 GenBank accession no. GU256055.
161
162
163 **Results and Discussion**
164
165 **Cloning and sequencing of pSP02**
166 The plasmid complement of *B. longum* M62 has been shown to be composed of three
167 plasmids of lengths around 3.5 (pSP01), 5.0 (pSP02) and 14.0 (pSP03) kbp (Álvarez-
168 Martín et al., 2007). Restriction analysis of the whole plasmid complement of strain
169 M62 showed pSP02 to be digested by *Bam*HI into two fragments of approximately 2.35
170 and 2.65 kbp; neither pSP01 nor pSP03 were cut with this enzyme. The digestion
171 fragments were ligated into *Bam*HI-digested pUC18, and the ligation mixture

electroporated into *E. coli* DH11S. Plasmid DNA from each of the clones was isolated, purified and sequenced. To check the sequence around the *Bam*HI positions, primers were designed to amplify segments flanking the *Bam*HI sites (Table 1). Sequencing of the amplicons confirmed that the whole molecule of pSP02 was composed of only these two *Bam*HI fragments, and to be 4896 bp long. The single *Kpn*I restriction enzyme site was used as a starting point for numbering nucleotides in a clockwise direction with respect to the ORF encoding the putative replicase RepA (Fig. 1). The GC content of the plasmid molecule was shown to be 62%, slightly higher than that recorded for the *B. longum* chromosome (60%) (Schell et al., 2002). It also showed a nucleotide sequence almost identical to that of other bifidobacterial plasmids, such as p6043A from *B. longum* DPC6043 (DQ458910), pB80 from *Bifidobacterium bifidum* B80 (DQ305402; Shokoporov et al., 2008), or pBIFA24 from *Bifidobacterium* spp. A24 (DQ286581). Less -but still extensive- homology was shown with the sequence of plasmids pKJ50 (BLU76614; Park et al., 1999), p157F-NC2 (AP010891) and pNAL8M (AM183144; Guglielmetti et al., 2007), and others. Furthermore, hybridization experiments using as a probe the whole *repA* gene amplified with primers RepAF and RepAR (Table 1) showed a replicon closely related to pSP02 to be present in certain plasmid-containing strains of our collection (data not shwon). Thus, a plasmid identical or very similar to pSP02 seems to be well spread among *B. longum* strains, and is also present in other bifidobacterial species (Shkoporov et al., 2008). As these plasmids are all cryptic, the biological significance of the presence of pSP02-type replicons is at present unknown. It may represent a very successful replicon in bifidobacteria, as is the case of WO02-type plasmids in *Lactococcus lactis* (Seegers et al., 1994).

Sequence analysis of pSP02

Four ORFs coding for proteins over 50 amino acids long were predicted into the pSP02 molecule (Fig. 1). Among the deduced proteins only a replicase (RepA) and a mobilization-like protein (MobA) showed any known functional domain. RepA was shown to consist of 303 amino acids long harboring a conserved replicase domain of the Rep_3 protein superfamily (pfam01051). BLAST analysis showed RepA to differ from the equivalent sequence of plasmid pB80 of *B. bifidum* B80 (Shkoporov et al., 2008) by a single amino acid, and by two amino acids with respect to pNAL8M (Guglielmetti et al., 2007). All these plasmids can be included in the phylogenetic group Ia of the bifidobacterial plasmids (Guglielmetti et al., 2007). The deduced MobA protein of pSP02 is thought to be composed of 370 amino acids. The protein contained a conserved domain of transfer relaxases of the MobA_MobL superfamily (pfam03389). The deduced amino acid sequence of two additional ORFs, *orf1* and *orf2*, showed no putative conserved domains and share no homology to proteins in the databases.

A large number of direct (DR) and inverted (IR) repeats were detected in the pSP02 sequence. The approximate position of all these are indicated in Fig. 1. Secondary structures of DRs and IRs were particularly abundant between *orf2* and *repA* genes. These secondary structures conform what it is supposed to be the origin of replication (*ori*) of pSP02, a region which is detailed in Fig. 2. In fact, the organization of this region resembles that found in the *ori* region of plasmids of Gram-positive bacteria in which the theta mode of replication has been experimentally demonstrated (Kiewiet et al., 1993; del Solar et al., 1998). Two AT-rich stretches of 30 and 27 nt were identified in this region, which are flanked by a typical DR of 22 nt (DR-6), repeated three times and featuring an iteron structure similar to that in pKJ50 (Park et al., 1997). The first and third repeats are identical and embrace an imperfect repeat in the middle (DR-6*)

(Fig. 2). Also noteworthy in the pSP02 sequence is the presence of a long IR of 38 nt, the two copies of which are separated by more than 900 bps (IR-2 in Fig. 1).

No canonical *oriT*-like sequence was identified in front of *mobA*. However, a short inverted repeat of eight nt (5'-ATGTTACC-GGT-GGTAACAT-3') separated by six bp from a 12 nt sequence (5′-TAAGTGCGCCCT-3′) resembling that present in some mobilizable plasmids from Gram-positive bacteria (Lee and O'Sullivan, 2006), was found through positions 3086 to 3050, 228 bp upstream of the start codon of *mobA*.

Construction of pSP02-derived vectors

Fig. 3 shows the general outline for the construction of the pSP02-derivatives. A clone in pUC18 carrying the *Bam*HI fragment encoding *repA* was digested with *Bam*HI and ligated into *Bam*HI-digested pUC19E, a pUC-derived vector unable to replicate in bifidobacteria but harboring the erythromycin resistance gene of pE194 from *Staphylococcus aureus*. The ligation mixture was electroporated into electrocompetent *Bifidobacterium pseudocatenulatum* M115 cells. Transformants carrying the pSP02 fragment in the two opposite orientations were found, showing that orientation was not essential for replication. It was also shown that neither *mobA* nor *orf1* nor the IRs present in the other half of the molecule was necessary for replication.

One construct carrying the replicase in the same relative orientation to that of the betalactamase gene in pUC19E, named pSP1 (Fig. 3), was selected and further modified. As shown in the figure, the erythromycin resistance gene in pSP1 was replaced by a bifidobacterial *tet*(W) gene encoding resistance to tetracycline (Álvarez-Martín et al., 2008), giving raise to pSP2 (Fig. 3). This construct was first obtained in *E. coli* and then transformed into *B. pseudocatenulatum*.

Finally, pSP1 was double digested with *Eco*RI and *Hin*dIII, treated with the Klenow fragment of *E. coli* DNA polymerase I, re-ligated and transformed into the M115 strain. Transformants lacking DNA from the pUC vector were recovered, thus demonstrating that the replication of this construct, called pSP3 (Fig. 3), was driven by sequences and elements from the pSP02 segment.

Host range, copy number and stability of pSP02 derivatives

A series of bifidobacterial strains belonging to different species, including recognized probiotic strains such as *Bifidobacterium animalis* subsp. *lactis* Bb12 and *Bifidobacterium breve* UCC 2003 (Table 2), were made competent and transformed with one μg of DNA from all pSP02-derived constructs (pSP1, pSP2, and pSP3). Though the transformation frequency varied widely among the different constructs and strains, transformants were recovered in all cases, except for pSP3, for which, as expected, transformants in *E. coli* were not obtained. As a general trend, pSP1 and pSP3 showed similar transformation frequencies in the different hosts, while fewer transformants were usually obtained for pSP2. Transformants were obtained at low frequency for pSP1 and pSP3 in a single strain of *Corynebacterium glutamicum*, which belongs to the same phylum as bifidobacteria (*Actinobacteria*). However, no transformants with pSP02-derivatives were recovered in several lactic acid bacterial strains (Table 2), suggesting the replicon is not functional in this bacterial group.

The relative copy number per chromosome equivalent of pSP02 was analyzed in its original host *B. longum* M62 by qPCR; those of the constructs were analyzed after transformation into the plasmid-free strain *B. longum* L25. The standard amplification curves both *repA* and *tuf* genes were linear over the tested range in both strains (average R2 >0.993). Assuming maximum and identical amplification efficiencies for the target

(*repA*) and reference (*tuf*) genes, the relative copy numbers per chromosome equivalent of pSP02 was estimated to be around 12 copies in both strains (11.83±0.64 in *B. longum* L25 and 12.39±0.92 in *B. longum* M62). A similar copy number was also established under the same experimental conditions for all pSP02-derivatives in *B. longum* L25. Copy number of pSP02 showed to be intermediate to that of pBC1 (30 copies per cell) (Álvarez-Martín et al., 2007) and pCIBAO89 (4 copies per cell) (Cronin et al., 2007).

pSP1, pSP2, and pSP3 were checked for stability under non-selective conditions in *B. pseudocatenulatum* M115. After 100 generations more than 80% of the colonies retained each of the constructs. Plasmid analysis by digestion with restriction enzymes showed that antibiotic resistant colonies contained the constructs without appreciable structural changes.

pSP1 was also electroporated into strain M115 containing pAM4, a cloning vector based on pBC1 from *B. catenulatum* L48 (Álvarez-Martín et al., 2008). The two plasmids remained compatible and were stably maintained at similar frequencies to those shown by each of the construct in this host.

Mode of replication of pSP02

As *B. longum* M62 carries three plasmids, to investigate the mode of replication of pSP02, total DNA was isolated from an overnight culture of *B. longum* L25 harboring vector pSP1. Purified untreated DNA and DNA treated with nuclease S1 were transferred to a membrane under non-denaturing conditions and hybridized with the above referred *recA*-derived probe. As can be seen in Fig. 4, pretreatment of the DNA with nuclease S1, resulted in the loss of a hybridization band which is thought to correspond to ssDNA. The detection of ssDNA as an intermediate in the replication of a plasmid is currently used as a proof of using the RC type of replication (del Solar et al.,

1998; Khan, 1997). The apparent discrepancy between the physical organization of the *ori* region of pSP02 (similar to theta-replicating plasmids) and the replication proceeding by the RC mode has previously been noted for other *B. longum* plasmids such as pKJ50 (Park et al., 1999) and pDOJH10L-RepB (Lee and O'Sullivan, 2006).

In conclusion, a cryptic plasmid from *B. longum* was analyzed and used for the construction of cloning vectors that replicate in different bifidobacterial species. Although presenting an iteron-like structure and other elements typical of theta-replicating plasmids (AT-rich regions separated by GC-rich stretches), pSP02 was shown to replicate through ssDNA intermediates, consistent with an RC mode. pSP02-derived constructs showed convenient stability and copy number. These new plasmid vectors could complement the genetic tools already available for performing molecular studies on bifidobacteria and their genetic manipulation.

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422 Yazawa, K., Fujimori, M., Nakamura, T., Sasaki, T., Amano, J., Kano, Y., Taniguchi,
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Table 1.- Bacterial strains, plasmids and oligonucleotide primers utilized in this work.

Item	Genotype, phenotype or sequence	Source or reference
Strains		
Escherichia coli DH11S	*mcrA,* Δ*(mrr-hsdRMS-mcrBC),* Δ*(lac-proAB),* Δ*(rec1398), deoR, rpsL, srl-thi-*/F'*proAB*+ *lacIqZ*ΔM15	Invitrogen
Bifidobacterium longum M62	Human intestinal isolate harbouring three cryptic plasmids, including pSP02	Álvarez-Martín et al. (2007)
B. longum C63	Human intestinal isolate harbouring two cryptic plasmids	Álvarez-Martín et al. (2007)
B. longum C72	Human intestinal isolate harbouring three cryptic plasmids	Álvarez-Martín et al. (2007)
B. longum L25	Human isolate, plasmid free	IPLA Laboratory Collection
Bifidobacterium adolescentis LMG 10502	Human intestinal isolate, plasmid-free	BCCM/LMG[1]
Bifidobacterium animalis LMG 10508	Human intestinal isolate, plasmid-free	BCCM/LMG
Bifidobacterium animalis subsp. *lactis* Bb12	Isolated from a fermented milk starter	Chr. Hansen, Denmark
Bifidobacterium breve LMG 13208	Human intestinal isolate, plasmid-free	BCCM/LMG
Bifidobacterium catenulatum L48	Human intestinal strain containing pBC1	Álvarez-Martín et al. (2007)
Bifidobacterium pseudocatenulatum M115	Human intestinal isolate, plasmid-free	IPLA Laboratory Collection
Bifidobacterium pseudolongum LMG 11571	Human intestinal isolate, plasmid-free	BCCM/LMG
Bifidobacterium thermophilus LMG 11571	Human intestinal isolate, plasmid-free	BCCM/LMG
Corynebacterium glutamicum LMG 19741	Plasmid-free	BCCM/LMG
Enterococcus faecalis ATCC 47077	Plasmid-free	ATCC[2]
Lactobacillus casei ATCC 393	Plasmid-free	ATCC
Lactococcus lactis subsp. *cremoris* MG 1363	NCDO 712 derivative, plasmid-free	Laboratory collection
Plasmids		
pUC18	Cloning vector, Ap^r, 2.686 Kbp	Yanisch-Perron et al. (1985)
pUC19E	pUC-based cloning vector, Ap^r Em^r, 3665 bp	Laboratory collection
pAM4	*E. coli-Bifidobacterium* shuttle vector; Ap^r Em^r Tet^r [tet(W)]	Álvarez-Martín et al. (2008)
pSP1	*E. coli-Bifidobacterium* shuttle vector; Ap^r Em^r, 6224 bp	This work
pSP2	*E. coli-Bifidobacterium* shuttle vector; Ap^r Tet^r, 7716 bp	This work
pSP3	*Bifidobacterium* vector, Em^r, 3594	This work
Oligonucleotides	(5'-3')	
Bam1F	GATGATCGGATGGAGCCTG (315 bp apart from Bam1R)	This work
Bam1R	CGATTGGCGTCGTCGATGG	This work
Bam2F	CACGGCTCACGTTCGACAC (275 bp apart from Bam2R)	This work
Bam2R	GTCCATGCACCGCTCTATGC	This work
RepAF	ATGTCCGATGAGATCGTGAAG	This work
RepAR	CGCAGCACTCGGCCAGTC	This work
CopyF	CACGTTCGAAGAATTGAAG (101 bp apart from CopyR)	This work
CopyR	GATGATGTCGTGCTCTTCG	This work
TufF	GGAGTACGACTTCAACCAG (110 bp apart from TufR)	This work
TufR	CATGTTCTTCACGAAGTCG	This work

Ap^r, Em^r, and Tet^r, resistance to ampicillin, erythromycin, and tetracycline, respectively.
[1]BCCM/LMG, Belgium Coordinated Collection of Microorganisms/Laboratory for Microbiology, University of Gent, Belgium
[2]ATCC, American Type Culture Collection.

Table 2.- Host range and transformation frequency of pSP02-derived vectors.

Species and strain	Transformants[1] per μg of DNA[2] of the vector		
	pSP1	**pSP2**	**pSP3**
B. adolescentis LMG 10502	$2.3x10^2$	$3.6x10^1$	$1.4x10^2$
B. animalis subsp. *animalis* LMG 10508	$7.4x10^1$	$5.0x10^0$	$5.8x10^1$
B. animalis subps. *lactis* Bb12	$1.8x10^1$	$6.0x10^0$	$3.0x10^1$
B. breve UCC 2003	$3.5x10^3$	$8.2x10^2$	$4.2x10^3$
B. longum L25	$6.5x10^1$	$3.0x10^0$	$2.3x10^2$
B. pseudocatenulatum M115	$1.2x10^4$	$6.3x10^3$	$4.6x10^5$
B. pseudolongum LMG 11571	$4.2x10^1$	$4.1x10^1$	$3.6x10^1$
B. thermophilus LMG 11571	$1.9x10^1$	$2.2x10^1$	$8.0x10^0$
Corynebacterium glutamicum LMG 19741	$1.6x10^1$	0	$5.0x10^0$
Enterococcus faecalis ATCC 47077	0	0	0
Lactobacillus casei ATCC 393	0	0	0
Lactococcus lactis subsp. *cremoris* MG 1363	0	0	0
Escherichia coli DH11S	$6.2x10^6$	$6.8x10^6$	0

[1]Results are the mean of two independent transformations.
[2]The DNA of all constructs was isolated from *B. pseudocatenulatum* M115.

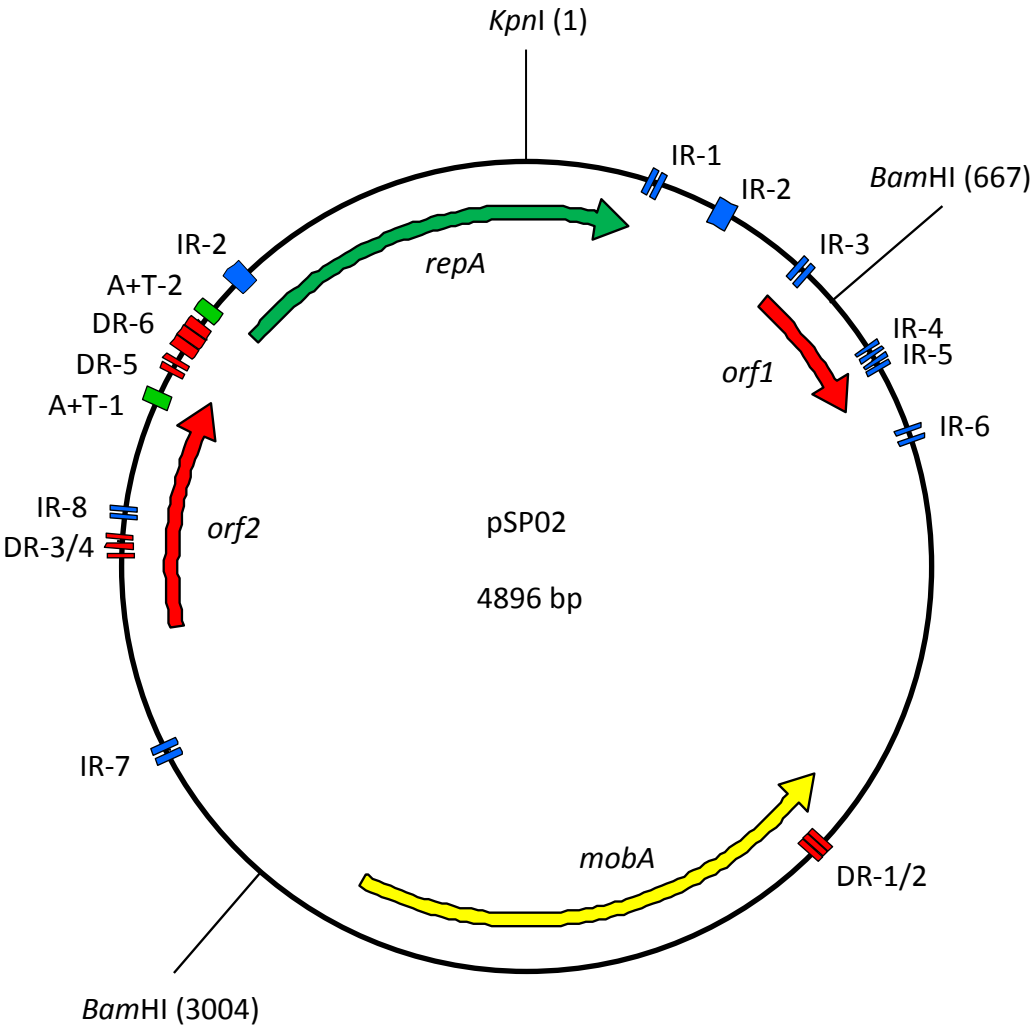
*Kpn*I (1)
IR-1
IR-2
*Bam*HI (667)
IR-3
IR-4
IR-5
IR-6
DR-1/2
mobA
*Bam*HI (3004)
IR-7
IR-8
DR-3/4
A+T-1
DR-5
DR-6
A+T-2
IR-2
repA
orf1
orf2
pSP02
4896 bp

Figure 1

Figure 2

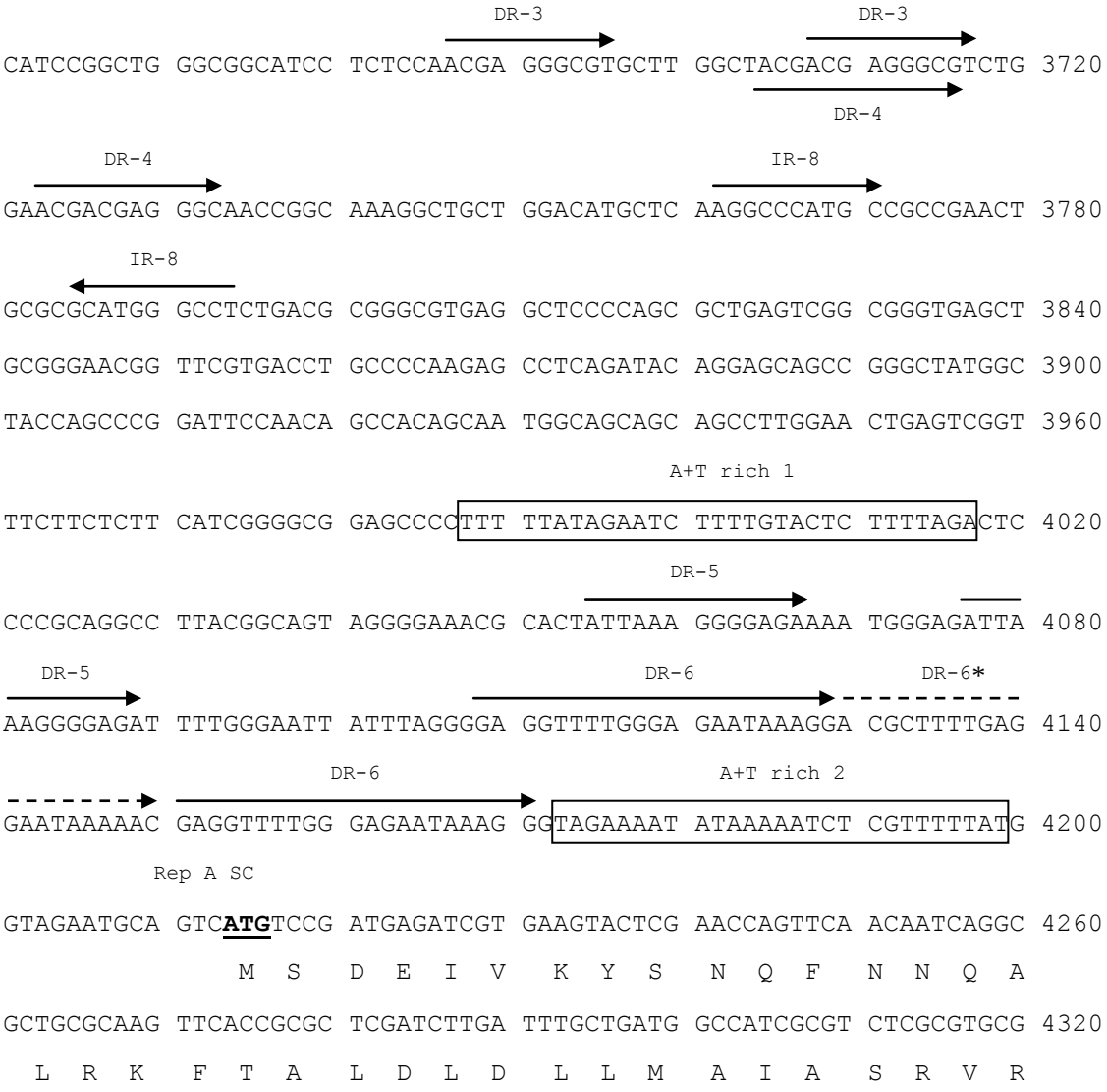

Figure 2

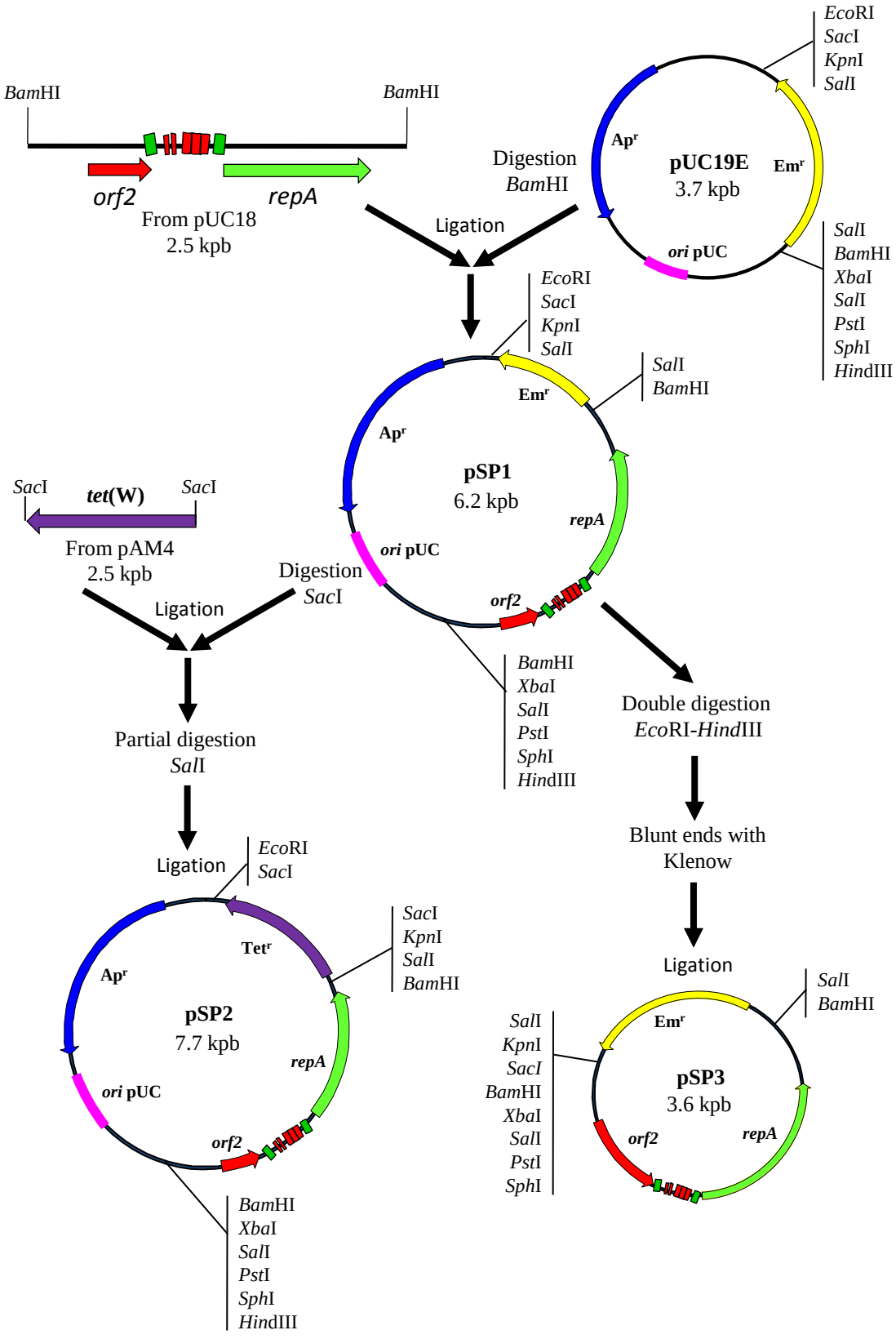
BamHI
BamHI
orf2
repA
From pUC18
2.5 kpb
Digestion
BamHI
Ligation
EcoRI
SacI
KpnI
SalI
Apr
pUC19E
3.7 kpb
Emr
ori pUC
SalI
BamHI
XbaI
SalI
PstI
SphI
HindIII
EcoRI
SacI
KpnI
SalI
SalI
BamHI
Emr
Apr
pSP1
6.2 kpb
repA
ori pUC
orf2
BamHI
XbaI
SalI
PstI
SphI
HindIII
SacI
tet(W)
SacI
From pAM4
2.5 kpb
Digestion
SacI
Ligation
Partial digestion
SalI
Double digestion
EcoRI-HindIII
Blunt ends with
Klenow
Ligation
EcoRI
SacI
Tetr
Apr
pSP2
7.7 kpb
repA
ori pUC
orf2
SacI
KpnI
SalI
BamHI
BamHI
XbaI
SalI
PstI
SphI
HindIII
Ligation
SalI
BamHI
Emr
pSP3
3.6 kpb
orf2
repA
SalI
KpnI
SacI
BamHI
XbaI
SalI
PstI
SphI

Figure 3

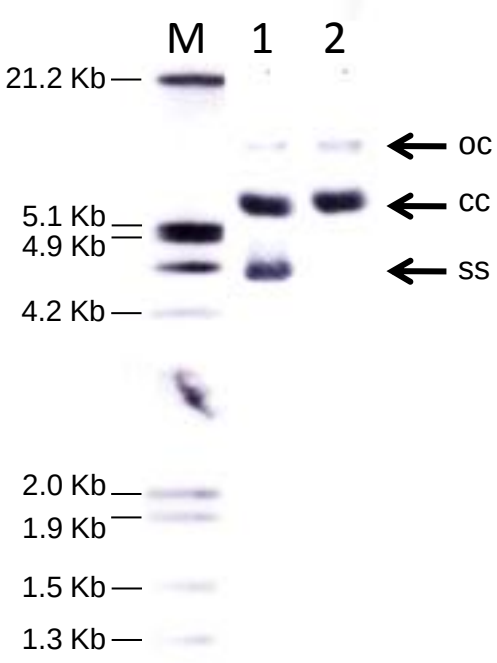

Figure 4

FIGURE LEGENDS

Figure 1.- Physical and genetic map of pSP02. The colored arrows indicate the direction and approximate length of the identified ORFs. Direct repeats (DRs) and inverted repeats (IRs) are indicated over the plasmid boundary by red- and blue-filled vertical blocks respectively. DR-1/2 and DR-2/3 indicate overlapping pairs of direct repeats. The two AT-rich regions are denoted by green-filled blocks. The position of the key restriction enzymes *Kpn*I and *Bam*HI sites is also indicated.

Figure 2.- Detailed DNA sequence of the *ori* region of pSP02 and the initial part of its corresponding *repA* gene, plus the latter's deduced amino acid sequence. Direct (DR) and inverted (IR) repeats are indicated by converging and same-direction arrows respectively; the numbering is as in Figure 2. The broken line indicates a copy of an imperfect DR6 repeat. The two AT-rich sequences are boxed.

Figure 3.- Diagram showing the construction of different pSP02-derived vectors. Ap^r, Em^r, and Tet^r indicate the ampicillin, erythromycin and tetracycline resistance genes respectively.

Figure 4.- Hybridization detection of ssDNA during the replication of pSP02 in *Bifidobacterium longum* L25 using a digoxigenin labeled *repA* amplicon as a probe. Line 1, purified, untreated DNA from whole-cell extracts; Line 2, DNA treated with nuclease S1. M, molecular weight marker, digoxigenin-labeled lambda DNA digested with *Eco*RI.