Stability of Recombinant Plasmids on the Continuous Culture of *Bifidobacterium animalis* ATCC 27536

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Abstract: *Bifidobacterium animalis* ATCC 27536 represents among bifidobacteria a host-model for cloning experiments. The segregational and structural stabilities of a family of cloning vectors with different molecular weights but sharing a common core were studied in continuous fermentation of the hosting *B. animalis* without selective pressure. The rate of plasmid loss *(R)* and the specific growth rate difference (δμ) between plasmid-free and plasmid-carrying cells were calculated for each plasmid and their relationship with plasmid size was studied. It was observed that both *R* and the numerical value of δμ increased exponentially with plasmid size. The exponential functions correlating the specific growth rate difference and the rate of plasmid loss with the plasmid molecular weight were determined. Furthermore, the smallest of the plasmids studied, pLAV (4.3-kb) was thoroughly characterized by means of its complete nucleotide sequence. It was found that it contained an extra DNA fragment, the first bifidobacterial insertion sequence characterised, named IS*1999*. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **84:** 145–150, 2003.
Keywords: plasmid stability; plasmid size; continuous fermentation; insertion sequence; bifidobacteria

INTRODUCTION

Bifidobacteria are predominant members of intestinal microflora and they exert a beneficial influence on the health of the host (Tannok, 1999). Strains of *Bifidobacterium animalis* are normally used as probiotics (Scalabrini et al., 1998). To enhance the probiotic characteristics of bifidobacteria a modification of the wild-type strain phenotypes by expression of heterologous genes, appears to be very promising. Recently, several efforts have been made to construct *Bifidobacterium* vectors (Rossi et al., 1996; 1998) and to develop efficient transformation protocols (Rossi et al., 1997). Furthermore, a few bifidobacterial genes were characterized and analyzed for their structure, organization, expression, and regulation (Minowa et al., 1989; Nunoura et al., 1996; Rossi et al., 2000).

Plasmid instability constitutes a major problem for industrial utilization of many recombinant microorganisms. It is essential that the vector, once introduced, can be stably maintained in the host strain, possibly even in absence of selective pressure. The controlled and constant environment provided by continuous cultures is an excellent tool for studying the effect of growth conditions on plasmid instability. Structural instability involves the rearrangement, loss or insertion of plasmid DNA sequences, associated typically with transposition or recombination. The segregational instability, which arises from a failure to distribute plasmid to both daughters at cell division, is affected by multiple factors including host and plasmid genotype and culture conditions. Previous studies have focused on culture conditions, demonstrating that segregational plasmid instability is affected by temperature, pH, aeration, composition of the growth medium, and dilution rate (Brigidi et al., 1997).

Among bifidobacteria *B. animalis* ATCC 27536 represents a host-model for cloning experiments because it is transformable by electroporation at relatively high efficiency (Rossi et al., 1997) and most of the vectors available for bifidobacteria have been constructed in this host (Rossi et al., 1996; Rossi et al., 1998).

In this study, the segregational instability of a family of recombinant plasmids, based on the common replicon pMB1 (Matteuzzi et al., 1990) and transformed in *B. animalis* ATCC 27536, was investigated.

MATERIALS AND METHODS

Bacterial Strain, Plasmids, and Media

Bifidobacterium animalis ATCC 27536 was used as cloning host to study the stability of the recombinant plasmids

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pLAV, pNC7, pLF5, pDG7, pLF5+2.3, and pLF5+6.4 in continuous fermentation. The construction of the *E. coli/Bifidobacterium* shuttle vectors pDG7 (7.2 kb) and pLF5 (5.4 kb), and of the *Bifidobacterium* vector pNC7 (4.9 kb) was previously reported (Matteuzzi et al., 1990; Rossi et al., 1998). The recombinant plasmids pLF5+2.3 (7.7 kb) and pLF5+6.4 (11.8 kb) were constructed by cloning into the *Escherichia coli/Bifidobacterium* shuttle vector pLF5, linearized with *Hin*dIII, the 2.3 and 6.4 kb fragments obtained by digestion of λ genomic DNA with *Hin*dIII. The attainment of the plasmid pLAV (4.3 kb) is described in this study.

Bifidobacterium animalis was subcultured anaerobically in MRS medium (Difco) containing 0.05% cysteine. Chloramphenicol (Cm) was added when required after sterilization to a final concentration of 5 μg/mL. Continuous fermentations were performed in a minimal glucose medium supplemented with 1.5% yeast extract, with composition (g/L^{-1}): glucose, 10; ascorbic acid, 10; sodium acetate, 10; yeast extract, 15; ammonium sulfate, 5; urea, 2; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $MnSO_4 \cdot 7H_2O$, 0.007; NaCl, 0.01; Tween 80, 1; cysteine, 0.5. The pH was adjusted to 6.0 with NaOH 4N. The media were autoclaved, 106°C, 30 min.

Fermentation Equipment and Growth Conditions

A BM-PPS3 3000 bioreactor (Bioindustrie Mantovane, Italy) with a working volume of 2 L was used under chemostat conditions, at a dilution rate of 0.36 h^{-1}. The bioreactor was thermostated at 38°C. Culture pH was maintained at a constant value of 6.0 by automatic titration with NaOH 4N. Constant stirring (400 rpm) was kept during the fermentation. Anaerobic conditions were maintained by using an oxygen-free mixture of CO_2+N_2 (3:7), which was sparged into the culture at a rate of 0.12 VVM and was flushed into the headspace of the feeding tanks. No antifoaming agents were used. The bioreactor was inoculated with a 10% (v/v) inoculum from a 24-h culture grown in MRS broth with added cysteine and chloramphenicol. Steady state was considered attained when the biomass concentration in the broth remained constant for at least two residence times. As a minimum, four residence times were allowed to elapse following a change of the cultivation conditions.

DNA Isolation, Manipulation, and Transformation

DNA manipulations were carried out according to standard procedures as described by Sambrook et al. (1989). DNA fragments from agarose gel were purified using the QiaexII Kit (Qiagen). *Bifidobacterium* plasmid extraction and electroporation were performed according to Rossi et al. (1996). To perform the plasmid extraction, cells from stationary phase were harvested by centrifugation and resuspended in TE buffer (Tris-HCl, 10 m*M;* EDTA, 1 m*M;* pH 8.0). The optical density was adjusted to an O.D. ($\lambda = 600$ nm) of 1.4, which corresponded to about 10^9 cells/mL^{-1}. Extraction of plasmid DNA from 10 mL aliquots of different samples and comparison of its signal intensity on agarose gels, allowed estimating the variation of plasmid copy number.

DNA Sequence Analysis

The nucleotide sequence of the plasmid pLAV was determined on both DNA strands using the DNA Sequencing Kit dRhodamine Terminator Cycle Sequencing ABI PRISM and the automatic sequencer ABI PRISM 310 Genetic Analyzer (PE Applied Biosystem). Every nucleotide was determined at least once on both strands (average: 2.3 times).

The sequence data obtained were compiled and analyzed on a UNIX computer using the Genetic Computer Group Programs Package (Madison, WI).

Stability of Recombinant Plasmids

Plasmid stability in *B. animalis* was studied in continuous cultures carried out without selective pressure, at pH = 6.0 and without control of CRP. Plasmid maintenance was estimated by cell counting on MRS agar plates. Samples were withdrawn at intervals from the continuous cultures, diluted, and then plated onto four MRS plates with and without antibiotic. One hundred single colonies were picked onto MRS Cm-containing plates and also onto MRS plates as a control. The average number of colonies on agar plates was estimated after anaerobic incubation at 37°C for 2 days. The number of colonies growing on the chloramphenicol plates was expressed as a proportion of the number growing on the control plates. This value was taken to represent the proportion of the plasmid-carrying population by its ability to form macroscopic colonies on the Cm-containing plates.

Kinetics of Plasmid Loss

The two kinetic and physiological variables which describe the generation of plasmid-free cell in a population of initially plasmid-carrying cells, are the rate of plasmid loss, R (gen^{-1}), and the specific growth rate difference between plasmid-free and plasmid-carrying cells, $\delta\mu$ (gen^{-1}). The kinetics of a system with unstable recombinant microorganisms has been described using models for growth competition. In a chemostat culture system it was shown (Cooper et al., 1987; Noak et al., 1984) that, assuming R and $\delta\mu$ to be constant, at time t—measured in units of generation time—the fraction of plasmid-free cells, y^-, is:

$$y^- = \frac{(y_0^- \cdot \delta\mu + R)e^{(\delta\mu+R)t} - R}{(y_0^- \cdot \delta\mu + R)e^{(\delta\mu+R)t} + \delta\mu} \quad (1)$$

where y_0^- is the fraction of plasmid-free cells at inoculation time ($t = 0$).

Equation (1) provides a relationship between the value of the experimentally assessable y^- and the two quantities R and $\delta\mu$. Because of the nonlinearity, it is not trivial to solve the equation to obtain an estimate for these two variables.

The model developed by Noak et al. (1984) and used by several authors (Brigidi et al., 1997) only considered the relationship between R and $\delta\mu$ when the specific growth rate difference was the dominant effect. A preliminary analysis of the data obtained in the present study showed that Noak's model did not work. In fact, the values of R and $\delta\mu$ calculated were in the relation $|\delta\mu| \leq R$ (Cooper et al., 1987). On the other hand, since y_0^- is usually very small, one can safely neglect the term $y_0^- \cdot \delta\mu$ compared to R in Eq. (1). Consequently:

$$y^- \cong \frac{e^{(\delta\mu+R)t} - 1}{e^{(\delta\mu+R)t} + \frac{\delta\mu}{R}} = 1 - \frac{\frac{\delta\mu}{R} + 1}{e^{(\delta\mu+R)t} + \frac{\delta\mu}{R}} \quad (2)$$

and thus,

$$y^+ \cong \frac{\frac{\delta\mu}{R} + 1}{e^{(\delta\mu+R)t} + \frac{\delta\mu}{R}} \quad (3)$$

Graphical analysis of the data showed that there was an exponential increase of y^- fraction in the chemostat. Here it can be assumed that

$$t >> \frac{1}{\delta\mu + R} \quad (4)$$

and therefore Eq. (3) can be reduced to:

$$y^+ \cong \left(1 + \frac{\delta\mu}{R}\right) e^{-(\delta\mu+R)t} \quad (5)$$

Plotting the natural logarithm of the fraction of plasmid-bearing cells against time (numbers of generations) a straight line is obtained with a negative slope m and intercept b. These can be used to calculate $\delta\mu$ and R:

$$m = -(\delta\mu + R) \quad (6a)$$

$$b = \ln\left(1 + \frac{\delta\mu}{R}\right) \quad (6b)$$

and hence:

$$\delta\mu = -m \cdot (1 - e^{-b}) \quad (7)$$

$$R = -m \cdot e^{-b} \quad (8)$$

Determination of Biomass Concentration

Biomass dry weight was determined by filtering the cells contained in 10 mL aliquots of fermentation broth onto preweighed membrane filters (0.45 μm), washing them with distilled water, drying at 100°C for 24 h and weighing. The net dry cell weight was obtained by subtracting the weight of the empty filters.

Statistical Analysis

Three independent experiments, i.e., continuous fermentations, were carried out with each plasmid studied. The cell counts were replicated four times. Data reported herein represent means. The 95% confidence limits on the parameters R and $\delta\mu$ were calculated using all possible combinations of the 95% confidence limits of the slope and intercept of the linear regression of ln y^+ vs. the number of generations, and considering as limits the maximum and minimum values obtained.

RESULTS

Attainment of pLAV and Nucleotide Sequence

The plasmid pLAV (4.3 kb) was obtained by transformation of *B. animalis* MB209 with the self-ligation mixture giving the recombinant plasmid pTRE3 (Rossi et al., 1998). In fact, while most of the Cm-resistant clones harbored a plasmid with the expected molecular weight (2.8 kb), one clone contained a larger plasmid (4.3 kb), named pLAV and characterized by analysis of its nucleotide sequence.

To determine the sequence, primers hybridizing on the *cat* gene were used and a primer-walking strategy was applied. The nucleotide sequence analysis revealed that the sequence of the parent vector pTRE3 was completely conserved, but 9-bp upstream from the −35 region of the *cat* promoter a 1402-bp foreign DNA fragment was present that showed the characteristic features of the insertion sequences (Guedon et al., 1995). It was named IS*1999*. The EMBL/GenBank accession number for this sequence is AJ318089.

The sequence of IS*1999* revealed in frame "a" the existence of a large open reading frame (*tnpA*), highly similar to other genes encoding transposases in *Mycobacterium smegmatis*, *Pseudomonas putida*, *Brevibacterium lactofermentum,* and *Corynebacterium glutamicum*. The gene *tnpA* (nt 133-1387) had a G+C content of 69.0% and was preceded by a polipurynic stretch (GGATGA) that could be a putative ribosome-binding site. The predicted gene product consisted of a 418 amino acids protein, with a calculated molecular mass of 47.5-kDa, A possible −10 consensus sequence of a putative promoter (TAGAGA, nt 53-58) could be found upstream from the *tnpA* gene. This motif presented good similarity with the corresponding structures found for the promoters of the *B. longum ldh* gene (TATAGA, Minowa et al., 1989), of the *B. longum lacZ* gene (TTATCC, Rossi et al., 2000) and of the operon encoding the replicative functions of plasmid pMB1 of *B. longum* MB203 (TATTCG, Rossi et al., 1996).

A second open reading frame (*orf*2) (nt 495-1392), could be located in frame "c" and overlapped entirely the *tnpA* gene. The predicted gene product consisted of a 299 amino acids protein, with calculated molecular mass of 34.3-kDa and isoelectric point 13.1.

A third open reading frame (*orf*3) (nt 839-488) could be located in frame "e," on the complementary strand and encoded a putative protein of 117 amino acids, with calculated molecular mass of 12.5-kDa and isoelectric point 12.5.

Segregational Instability of Recombinant Plasmids

In the first series of experiments, the segregational instability of recombinant plasmids pLAV, pNC7, pLF5 and pDG7 was studied. The results of these experiments showed a correlation between instability and plasmid size. To look further into the influence of plasmid size on the segregational instability, two larger plasmids, namely pLF5+2.3 and pLF5+6.4, were constructed by cloning into pLF5 the 2.3 and 6.4 kb fragments of λ genomic DNA. The natural logarithm of the fraction of plasmid-carrying cells (y^+) is in Figure 1 plotted against the number of generations for the six plasmids studied. In Figure 1 the linear regression lines are also shown with the respective correlation coefficients. All six regression lines were statistically significant with calculated *p*-values <0.0001 in all cases. The smallest plasmid studied, pLAV showed very high stability at the conditions used in the experiments. For the other plasmids, the decrease of y^+ was faster as the vector molecular weight increased. Still, it is worth noting the long plateau (from 0 to 30 generations) of y^+ values close to 1 and gentle slope

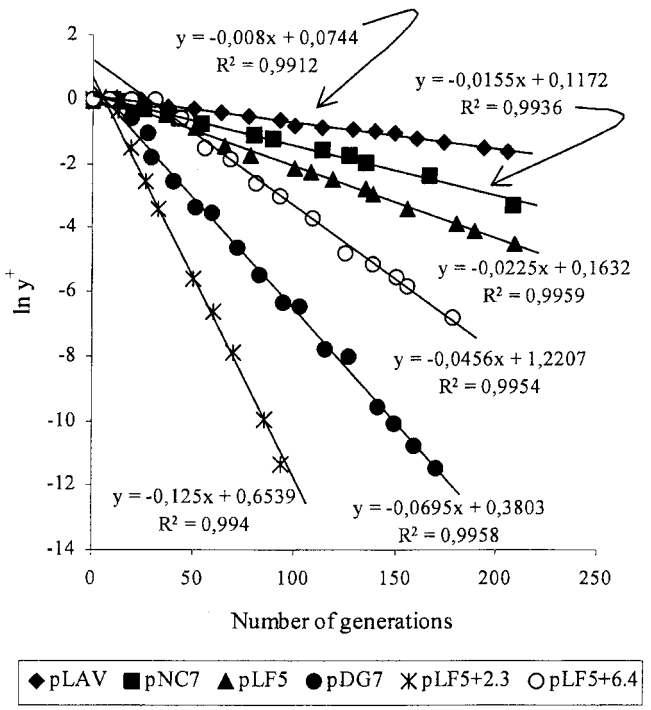

Figure 1. Regression lines of the natural logarithm of the fraction of plasmid-bearing cells (y^+, dimensionless) with respect to the fermentation time (number of generations) during continuous cultures of *B. animalis* transformed with pLAV (◆), pNC7 (■), pLF5 (▲), pDG7 (●), pLF5+2.3 (✱) or pLF5+6.4 (○), with the respective regression equations; R^2: correlation coefficient.

observed for pLF5+6.4 which was the largest plasmid among those studied. Applying the model previously ascribed to the plots on Figure 1, the rate of plasmid loss, *R* (gen^{-1}), and the specific growth rate difference between plasmid-free and plasmid-carrying cells, δμ (gen^{-1}) were calculated for each plasmid. The results obtained are shown in Table I. The values confirmed that the stability of the vectors was influenced by their size, with the exception of pLF5+6.4. In fact, the smallest plasmid pLAV (4.3 kb) exhibited the lowest values of *R* and δμ ($5.75 \cdot 10^{-4}$ and $75.66 \cdot 10^{-4}$ gen^{-1}, respectively). The 95% confidence limits on the parameters *R* and δμ were calculated using all possible combinations of the 95% confidence limits of the slope and intercept obtained from the regression lines shown in Figure 1, and the maximum and minimum results were considered as the limits; due to the complex formulae relating *R* and δμ to the slope and intercept of the regression lines, the 95% confidence limits on *R* and δμ are not symmetric about the predicted value.

Twenty-five single colonies of *B. animalis* MB209, selected on Cm-plates every 12 hours for each fermentation, were analyzed for the plasmid content. It was found that they all harbored the corresponding plasmid with the expected size and restriction pattern, indicating that deletion and rearrangement events had not occurred. Random analysis of colonies selected on Cm free plates showed the lack of recombinant plasmids. Therefore, the sensibility to Cm was due to plasmid loss and not to structural rearrangements compromising the phenotypic expression of Cm resistance gene.

Mini-prep DNA preparation of the samples tested during the continuous fermentations showed an electrophoretic plasmid signal of the same intensity, suggesting that the plasmid copy number did not change substantially during the fermentation.

DISCUSSION

To evaluate the plasmid maintenance in continuous fermentations of *B. animalis* ATCC 27536 without selective pres-

Table I. Calculated values of the rate of plasmid loss (*R*, gen^{-1}) and the specific growth rate difference between both plasmid-free and plasmid-carrying cells, (δμ, gen^{-1}) obtained during continuous cultures of *B. animalis* transformed with pLAV, pNC7, pLF5, pDG7, pLF5+2.3, or pLF5+6.4, with the respective 95% confidence limits (95% c. l.).

Plasmid	δμ	±95% c. l.	*R*	±95% c. l.
pLAV	0.00058	+0.00041 −0.00039	0.00745	+0.00078 −0.00073
pNC7	0.00171	+0.00094 −0.00092	0.01378	+0.00153 −0.00140
pLF5	0.00339	+0.00184 −0.00187	0.01909	+0.00255 −0.00227
pDG7	0.022	+0.01150 −0.01354	0.04754	+0.01539 −0.01167
pLF5+2.3	0.06	+0.02783 −0.03625	0.065	+0.04096 −0.02529
pLF5+6.4	0.03215	+0.00459 −0.00508	0.01345	+0.00455 −0.00342

sure, a family of plasmids derived from vector pLF5 (Rossi et al., 1998) was constructed. The members of this family pLF5, pLF5+2.3, and pLF5+6.4, together with the plasmids pLAV, pNC7, and pDG7 were transformed in *B. animalis,* enabling the comparison of the segregational and structural instabilities of similar plasmids differing above all for the molecular weight. The smallest of these plasmids, pLAV (4.3-kb), obtained accidentally, contained an extra DNA fragment consisting in the first bifidobacterial insertion sequence characterized, named IS*1999*. The analysis of IS*1999* showed the following characteristics of an IS element: It is flanked by short direct repeats which could result from a 7 bp duplication of target sequence at the insertion site during the transpositional event; *IS 1999* ends with 25-bp terminal inverted repeats with one mismatch; the amino acid sequence of the putative protein encoded by *tnpA* gene shares similarities with putative transposases of previously identified IS elements, in terms of size and average amino acid sequence. The predicted protein Orf2 was unusually abundant in arginine, hystidine, and proline and displayed sequence similarity of 49.1% with the product of the gene *orf*3 of *Haloferax* sp. (Holmes and Dyall-Smith, 1991), that overlaps in a different frame the gene *gyr*B encoding the DNA gyrase B subunit. The high content of positively charged residues of Orf2 and the similarity with a protein probably involved in DNA based mechanisms is suggestive of a DNA-binding protein which is probably involved in the transposition mechanism. The predicted protein Orf3 presented sequence similarity of 39.4% with the protein encoded by the *mob*B gene of *Escherichia coli* (Van Putten et al., 1987), suggesting the presence of a conjugative mechanism connected with the transposition modalities. A potential application of IS*1999* can be the development of a *Bifidobacterium* mutagenesis system.

As far as the segregational instability is concerned, the results of R and $\delta\mu$ obtained showed, with the exception of pLF5+6.4, a significant influence of plasmid molecular weight on the maintenance of the vectors assayed. In view of this result, a plot of R and $\delta\mu$ against plasmid size was made (Fig. 2). From the analysis of this plot, it is possible to calculate two exponential functions to correlate R and $\delta\mu$ with plasmid molecular weight for the five vectors considered, with respective correlation coefficients of 0.985 (p-value = 0.0008) and 0.989 (p-value = 0.0005). The influence of plasmid size on segregational instability has been previously reported (Cheah et al., 1987; Ostroff and Pene, 1984; Tanaka and Sueoka, 1983). Bron and Luxen (1985) proposed the reduction in copy number as the major factor in the size-dependent instability. As reported in the Results section, the constancy of plasmid signal intensity on agarose gel suggested that plasmid copy number did not change significantly during the continuous fermentations. The topological constraints that may prevent efficient membrane binding of the larger plasmids could be a plausible mechanism to explain the results obtained in this work. On the other hand, Corchero and Villaverde (1997) studied the plasmid maintenance in *E. coli* of a family of closely related recombinant vectors, and affirmed that plasmid maintenance decreased with the length of a cloned insert rather than with the size of the complete vector. The close correlation between plasmid size and the kinetics of plasmid loss observed in the present work allows considering the molecular weight of the vector as a direct factor influencing the plasmid maintenance.

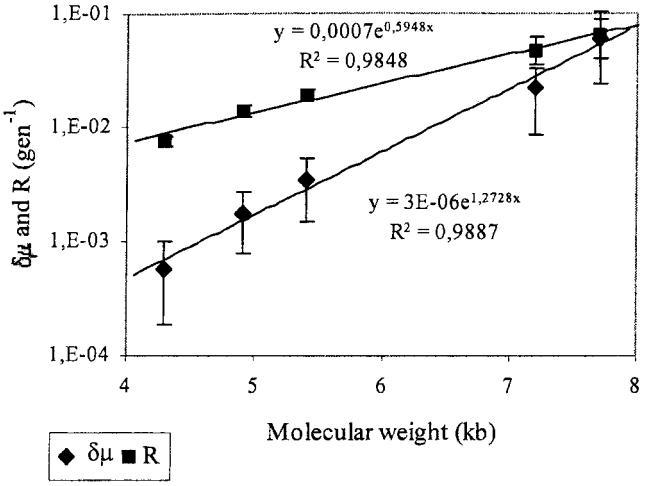

Figure 2. The rate of plasmid loss (R, gen^{-1}, ■) and the specific growth rate difference between both plasmid-free and plasmid-carrying cells, ($\delta\mu$ gen^{-1}, ◆) vs. plasmid molecular weight (kb), with the respective regression equations; R^2: correlation coefficient. Vertical segments represent 95% confidence limits. Note logarithmic scale in ordinate axis.

As previously mentioned, the values of R and $\delta\mu$ calculated for the largest plasmid, pLF5+6.4, mismatched the results obtained for the other five plasmids studied: even if $\delta\mu$ of pLF5+6.4 was of the same order of magnitude as that of the closest plasmids in size, pLF5+2.3 and pDG7, its value of R was surprisingly low for such a large plasmid. The initial delay in appearance of plasmid-free cells decreased the probability of plasmid loss estimated by the model, but once a significant amount of segregant cells arose, the kinetics of plasmid loss was similar to what might be expected (Fig. 1). The very low segregational stability displayed by pLF5+6.4 could probably be ascribed to modifications in its secondary structure that would have enabled an exceptionally high rate of plasmid replication, but once plasmid free cells began to emerge, the high kinetic advantage of segregant over transformant population would have led to a rapid washout of the transformant in the continuous culture by simple competition.

On the other hand, it is worth pointing out that no structural instability was observed for all the plasmids studied despite the long-lasting continuous fermentations carried out, indicating that the core of these vectors, consisting of the replicon pMB1 and of the *cat* gene of pC194 (Horinouchi and Weisblum, 1982), is suitable for cloning of foreign DNA in bifidobacteria.

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