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# The broad host range plasmid pLF1311 from *Lactobacillus fermentum* VKM1311

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**Abstract**

The complete nucleotide sequence (2389 bp) of the cryptic plasmid pLF1311 from *Lactobacillus fermentum* VKM1311 was determined. DNA sequence analysis revealed the putative coding regions for a replicative protein (RepB), its repressor (RepA) and double-stranded (*dso*) and single-stranded (*sso*) origins. pLF1311 belongs to the pE194 family of rolling circle-replicating plasmids. A derivative of pLF1311 that contains the *cat* gene of plasmid pC194 of *Staphylococcus aureus* and the *oriT* of RP4 was constructed and transferred by conjugative mobilization from *Escherichia coli* to various Gram-positive bacteria. The stable maintenance of this derivative was shown in some strains of *Lactobacillus, Lactococcus, Enterococcus* and *Bacillus* under non-selective conditions. 

*Keywords:* Rolling circle replication; Mobilizable vector; *Lactobacillus* plasmid

## 1. Introduction

Lactobacilli are Gram-positive bacteria that have been used in the production of various kinds of food over centuries. Some *Lactobacillus* strains may have a significant general beneficial effect on human and animal health and therefore, they may be used as probiotics [1].

Many *Lactobacillus* species contain one or more plasmids. Some of them were analyzed at the molecular level ([2] and references therein, [3,4]). All these plasmids have been shown to replicate by the rolling circle (RC) mechanism that includes the formation of single-stranded DNA (ssDNA) intermediates. RC plasmids constitute a group of small, multicopy replicons that have been widely spread among bacteria (see [5,6] for review). The indispensable elements of RC plasmids are a gene for the replicative protein (Rep) controlled by a repressor and the plus origin of replication (*dso*). In addition, almost all RC plasmids contain the minus origin of replication (*sso*).

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Regarding the *dso* and Rep homology, plasmids have been classified into several families. The known *Lactobacillus* plasmids belong to either pE194 or pC194 families [2].

RC plasmids usually have a broad host range. They are suitable for vector constructing and gene cloning [2,6].

In this study, we determined the nucleotide sequence of a new cryptic plasmid (designated pLF1311) from *Lactobacillus fermentum* VKM1311. We identified structural elements of RC plasmids in pLF1311 and tested the plasmid for its replication ability in different host cells.

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 1. The strains of *Escherichia coli* and *Bacillus* were grown in Luria-Bertani (LB) broth under continuous agitation or on LB agar [7] at 37°C. The strains of *Lactobacillus* were grown statically at 37°C in MRS medium (Difco, USA). *Lactococcus lactis* was grown statically at 30°C in M17 medium [12]. For selection, the media were supplemented with the appropriate antibiotics using the following concentrations (μg $ml^{-1}$): ampicillin (Ap), 100; chloramphenicol (Cm), 10; streptomycin (Sm), 100; kanamycin (Km), 50 and polymyxin (Pm), 10.

Table 1
Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *Bacillus amyloliquefaciens* B1796 | $Sm^r$ $Pm^r$ | VKPM[a] |
| *B. subtilis* B1727 $Sm^r$ | $Sm^r$ $Pm^r$ | Spontaneous $Sm^r$ mutant of B1727 (VKPM) |
| *Bacillus thuringiensis* ssp. *galleriae* B1164 | $Pm^r$ | VKPM |
| *B. thuringiensis* ssp. *finitimus* B1163 | $Pm^r$ | VKPM |
| *B. thuringiensis* ssp. *kurstaki* B3847 | $Pm^r$ | VKPM |
| *B. flavum* B120 | $Pm^r$ | VKPM |
| *Enterococcus faecalis* OG1 | $Sm^r$ $Pm^r$ | V.A. Livshits collection |
| *Enterococcus faecium* M74 | $Pm^r$ | V.A. Livshits collection |
| *E. coli* C600 | [7] | |
| *E. coli* HB101 | $Sm^r$ | [7] |
| *E. coli* TG1 | [7] | |
| *Lactobacillus brevis* VKM1309 | $Sm^r$ $Pm^r$ | VKM[b] |
| *Lactobacillus buchneri* VKM1310 | $Pm^r$ | VKM |
| *Lactobacillus casei* VKM535 | $Pm^r$ | VKM |
| *L. fermentum* VKM1311 | $Pm^r$ | VKM |
| *L. plantarum* VKM578 | $Pm^r$ | VKM |
| *L. lactis* LPIT6 | $Sm^r$ $Pm^r$ | L.L. McKay |
| pSUP5011 | $Ap^r$ $Cm^r$ *oriT* | [8] |
| RP4 | $Ap^r$ $Km^r$ $Tc^r$ $Tra^+$ | [9] |
| pUC4K | Cloning vector $Ap^r$ $Km^r$ | |
| pUC19 | Cloning vector $Ap^r$ | |
| pC194 | $Cm^r$ | [10] |
| pLF1311 | Cryptic plasmid from *L. fermentum* VKM1311 | This study |
| pLF2 | $Cm^r$, pLF1311::(1.1 kb of pC194) | This study |
| pLFVM2 | $Cm^r$, *oriT*, pLF2::(1.5 kb *BamH*I of pSUP5011), Δ0.15 kb | This study |
| pLFD1 | $Cm^r$, pLFVM2 Δ2.4 kb *Sal*I | This study |
| pLFA2 | $Ap^r$ $Cm^r$ | [11] |

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[b]Russian Collection of Microorganisms.


Fig. 1. Alignment of (A) RepA proteins and (B) RepB proteins of LF1311 and pLB4. An asterisk indicates the residues shared by both sequences (identical residues) and a point indicates similar residues.

### 2.2. Mating procedure

Plasmid pLFVM2 was transferred by conjugation from the donor C600 (RP4, pLFVM2) into different bacteria using filter matings [13]. Sm or Pm were used for counter-selection of the donor. The segregational stability of plasmids in different hosts was determined as described by Posno et al. [14].

### 2.3. DNA techniques and plasmid transformation

Plasmid DNA from *E. coli* and *Bacillus* strains was extracted by the alkaline lysis method [7]. Plasmid DNA of *Lactobacillus* strains was prepared as described by Klaenhammer [15]. Molecular cloning techniques were applied according to Sambrook et al. [7]. Plasmid transformation of *Bacillus subtilis* was performed according to Anagnostopoulos and Spizizen [16]. DNA fragments of pLF1311 were cloned into the pUC19 vector and the nucleotide sequence was determined by the dideoxy-chain termination method [17]. The sequence data were analyzed using DNASUN software [18].

The DNA sequence revealed in this study is given by the accession number X74860 of the EMBL data library.

## 3. Results and discussion

### 3.1. DNA sequence of pLF1311 and the prediction of coding regions

*L. fermentum* VKM1311 possesses only one cryptic plasmid, namely, the plasmid pLF1311. The complete nucleotide sequence of this plasmid (3289 bp) was determined. The calculated GC content of the pLF1311 plasmid (36.0%) is more typical of some *Streptococcus* species or *L. lactis* [19] than of *L. fermentum* (52–54%) [20]. Two open reading frames (ORFs) were identified within the pLF1311 sequence. These ORFs are oriented in the same direc-

tion and are separated by 76 bp. ORF1 consists of 156 bp, it was deduced to encode a polypeptide with a molecular mass of 6 kDa. ORF2 consists of 681 bp and is able to encode a polypeptide with a molecular mass of 27 kDa. Both protein coding regions are assumed to have ATG initiation codons. Potential ribosome-binding sites (SD) were identified upstream of each ORF. The SD boxes for ORF1 (AGAAGG) and ORF2 (GAGG) were located at a position of 7 and 12 bp upstream of the corresponding initiation codons. A potential promoter sequence located upstream of ORF1 was found (the region of −35: 5′-TTGTAT-3′ and the region of −10: 5′-TATAAT-3′). A search for homology with the deduced protein sequences of ORF1 and ORF2 in sequence databases revealed that they are related to the proteins encoded by RC plasmids of the pE194 family. The protein products of ORF1 and ORF2 from pLF1311 are homologous to the RepA (98% amino acid identity) and the RepB (90% amino acid identity) proteins of pLB4 from *Lactobacillus plantarum* [21] (Fig. 1). According to this homology, RepB and RepA were identified as a replicative protein and its gene repressor, respectively [5,6,21]. The RepB protein of pLF1311 is also similar to that of pE194 from *Staphylococcus aureus* and pLA106 from *Lactobacillus acidophilus* (41% amino acid identity). No significant homology was found in non-coding regions of pLF1311 and other plasmids, except for *dso* and *sso* regions.

### 3.2. Analysis of pLF1311 non-coding regions

It is known that the *dso* region of RC plasmids contains functional elements required in *cis* for the initiation of the leading strand synthesis [5,6]. The nicking site (*nic*), from which replication starts, is the most important part of the *dso* region and is highly conserved among plasmids belonging to the same family [5]. Based on this homology, we identified the *nic* site sequence 5′-CACTACGA-3′ upstream of ORF1 in pLF1311. This *nic* site located in the loop of the potential hairpin structure (Fig. 2A) is identical to that of pA1 from *L. plantarum* and differs in a single nucleotide from the *nic* consensus of the pE194 family: 5′-TACTACG/A-3′.

The conversion of ssDNA into double-stranded (ds) DNA initiates at the *sso* site. The potential *sso* region with a specific hairpin structure was identified upstream of *dso* (Fig. 2B). The imperfect inverted repeats of *sso* that form the palindrome are highly conserved in all *Lactobacillus* plasmids studied [22] and pLF1311 is not an exception.

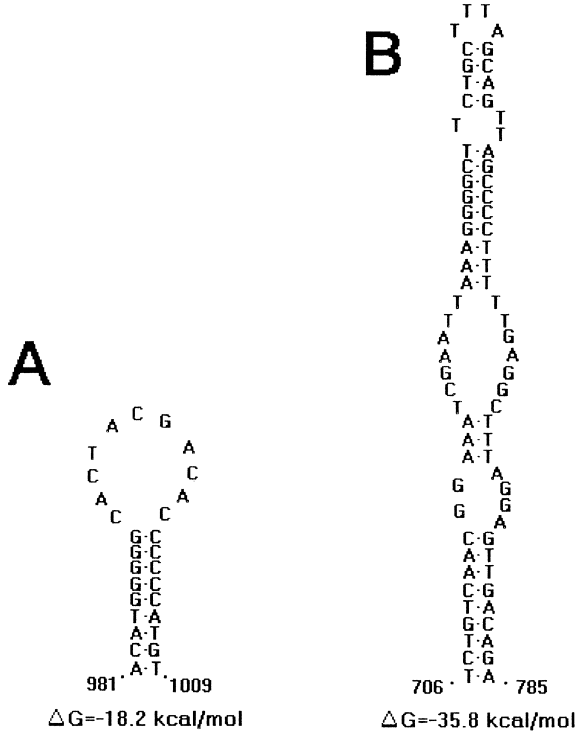


Fig. 2. The regions of (A) *dso* and (B) *sso* of pLF1311. For each secondary structure, the difference in the free energy of hairpin formation is shown below.

### 3.3. Replication of the pLF1311 in E. coli and identification of the replication region

In order to examine whether pLF1311 can be maintained in *E. coli* cells, pLF1311 was marked by the *cat* gene ($Cm^r$) from pC194. The resulting plasmid pLF2 was selected in *B. subtilis* cells. Next, it was transformed into *E. coli* C600 cells and $Cm^r$-resistant colonies were easily obtained.

To define the minimal region of pLF1311 that is essential for its replication, several deletion and insertion variants of pLF2 were obtained and tested for their replication ability in the *E. coli* C600 trans-

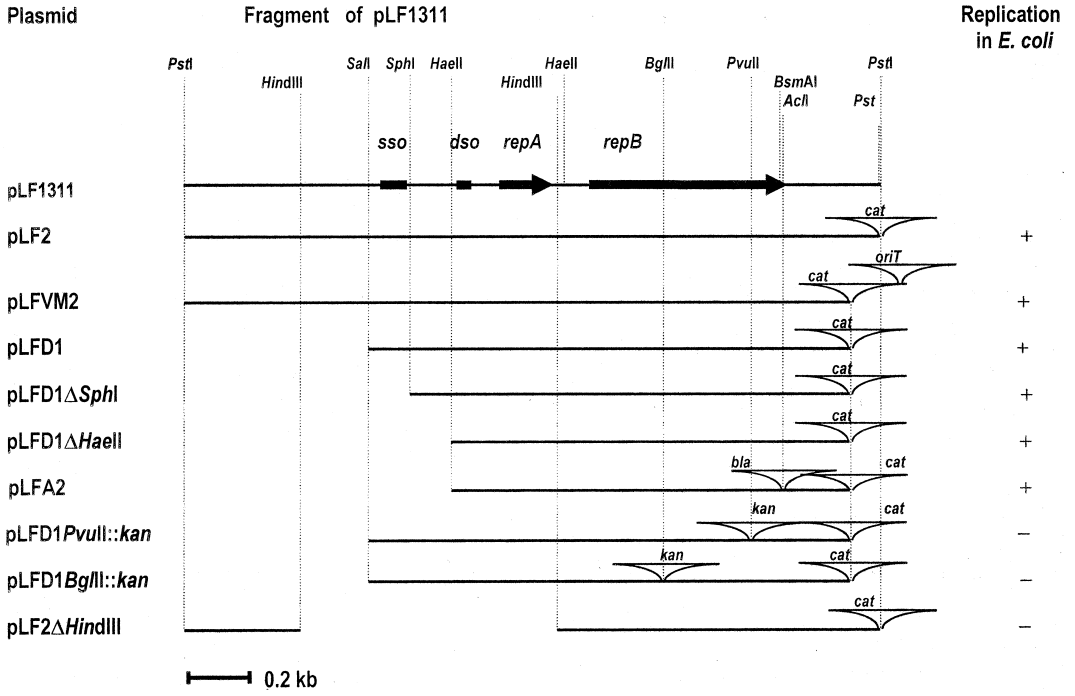


Fig. 3. Deletion and insertion mapping of the replication region of the pLF1311 plasmid. The construction of pLF2, pLFVM2, pLFD1 and pLFA2 is described in Table 1. The source of the *kan* gene was pUC4K. The replication ability was considered positive (+) when confirmed transformants were obtained and negative (−) when no transformants were obtained in three experiments.

formants. As can be seen in Fig. 3, plasmid pLFD1Δ*Hae*II contains the minimal part of pLF1311 that includes the *dso* region and the *repA* and *repB* genes. The *sso* region seems to be a dispensable element for pLF1311 replication. Conversely, the *dso* region is an obligatory element. Therefore, the pLFD1Δ*Hin*dIII plasmid could not be replicated in *E. coli* C600 cells (Fig. 3). It is evident that the *repB* gene product is essential for pLF1311 replication. The pLFD1 derivatives, pLFD1*Pvu*II::*kan* and pLFD1*Bgl*II::*kan*, were found to lose the ability of autonomous replication because of the disruption of *repB*. However, these plasmids could be maintained in the cells that possess an intact *repB* gene in another replicon (data not presented). Interestingly, insertion of the *bla* gene into the *Acl*I restriction site, which substitutes 10 C-terminal amino acid residues of RepB, was shown to decrease the growth rate of *E. coli* cells in an antibiotic-containing medium (plasmid pLFA2, Fig. 3) [11].

### 3.4. Host range of pLF1311

To study the host range of the pLF1311 replicon, plasmid pLFVM2 was constructed. The plasmid was transferred by conjugative mobilization from *E. coli* to various Gram-positive bacteria (Table 2). Plasmid pLFVM2 is characterized by stable maintenance in the majority of Gram-positive bacteria under non-selective conditions. In *Brevibacterium flavum* and *E. coli*, it was found to be unstable without selection (Table 2). Data on agarose the gel mobility and restriction pattern suggest that after 20 generations of non-selective growth, pLFVM2 was structurally unaltered, regardless of the host from which it was prepared for analysis.

Thus, the potential host range of pLF1311 is very broad. This feature is characteristic of many other RC plasmids [5,6]. It seems to be a result of their rational replication mode. RC plasmids exploit a host replicative system. The plasmid-encoded Rep

Table 2
Conjugative transfer of pLFVM2 from *E. coli* into different bacteria and segregational stability of the corresponding transconjugants

| Recipient strain[a] | Frequency of transfer[b] | Stability[c] |
|---|---|---|
| *L. brevis* VKM1303 | $3\times10^{-8}$ | 100 |
| *L. buchneri* VKM1310 | $1\times10^{-7}$ | 100 |
| *L. lactis* LPIT6 | $3\times10^{-7}$ | 100 |
| *E. faecalis* OG1 | $1\times10^{-5}$ | 100 |
| *E. faecium* M 74 | $5\times10^{-7}$ | 100 |
| *B. subtilis* B1727 $Sm^r$ | $5\times10^{-5}$ | 96 |
| *B. thuringiensis* ssp. *galleriae* B1164 | $2\times10^{-3}$ | 96 |
| *B. thuringiensis* ssp. *kurstaki* B3847 | $5\times10^{-6}$ | 56 |
| *B. thuringiensis* ssp. *finitimus* B1163 | $5\times10^{-5}$ | n.e.[d] |
| *B. amyloliquefaciens* B1796 | $2\times10^{-6}$ | 98 |
| *B. flavum* B120 | $5\times10^{-8}$ | 4 |
| *E. coli* HB101 | $1\times10^{-2}$ | 26 |

[a]We failed to introduce pLFVM2 into cells of the following bacteria: *Lactobacillus amylophilus*, *Lactobacillus delbrueckii*ssp. *bulgaricus*, *L. casei*, *Leuconostoc mesenteroides*, *Pediococcus* sp., *Streptococcus salivarius* ssp. *thermophilus*, *Micrococcus* sp., *S. aureus*, *Bacillus coagulans*, *Bacillus licheniformis* and *Bacillus stearothermophilus*.
[b]The frequency of transfer is the number of transconjugants per donor cell.
[c]The stability of pLFVM2 in the corresponding strains implies the percentage of chloramphenicol-resistant CFU after 20 generations of non-selective growth.
[d]Not estimated.

protein only introduces a nick in the corresponding plasmid site, from which the host cell enzymes start the replication. Therefore, the functioning promoter and SD box of the *repB* gene provide the maintenance of the plasmid in any potential host cell.

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