Microbiology, Vol. 69, No. 1, 2000, pp. 63–67. Translated from Mikrobiologiya, Vol. 69, No. 1, 2000, pp. 75–80.

EXPERIMENTAL ARTICLES

A Family of Shuttle Vectors for Lactic Acid Bacteria and Other Gram-Positive Bacteria Based on the Plasmid pLF1311 Replicon

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Received December 28, 1998

Abstract—A set of broad-host-range single-replicon shuttle vectors for cloning nucleotide sequences in gram-positive bacteria (lactobacilli, enterococci, lactococci, bacilli, etc.) was created. The vectors are based on the cryptic plasmid pLF1311 from *Lactobacillus fermentum* VKM 1311, belonging to the family of the σ-type pE194-like plasmids. The vectors can replicate in gram-positive bacteria and *Escherichia coli*. They are stable in many gram-positive bacteria, have small sizes, and allow the selection of recombinants on media with X-Gal. The vectors that contain the region of initiation of the conjugal transfer of plasmid RP4 belonging to the incompatibility group IncPα can be mobilized in a great number of bacteria using a helper plasmid from *E. coli* but not from gram-positive bacteria.

Key words: shuttle vectors, ss-DNA plasmids, gram-positive bacteria, lactic acid bacteria.

Lactic acid bacteria (lactobacilli, lactococci, enterococci) are of much practical significance. These important symbionts of humans and animals [1] are widely used in the food industry, in agriculture, veterinary practice, and medicine [2–4]. The strains used so far were mainly isolated from natural sources, whereas genetically engineered lactobacilli are still rare. In particular, there are no generally recognized vectors for the introduction of recombinant DNA into the aforementioned bacteria, although a number of plasmid vectors [5–11] and integration vectors [12–15] have been proposed for this purpose.

The aim of this work was to construct plasmid vectors based on the natural plasmid pLF1311 from *Lactobacillus fermentum* VKM 1311, which would be appropriate to gene cloning in lactic acid bacteria.

MATERIALS AND METHODS

Plasmids, strains, and cultivation conditions. Plasmids used in this study were derived from the natural cryptic plasmid pLF1311 of *Lactobacillus fermentum* VKM 1311, whose total nucleotide sequence has been submitted to the GenBank/EMBL database under accession number X74860. The derivatives of pLF1311 were marked by the chloramphenicol resistance gene from the staphylococcal plasmid pC194 [16] or the kanamycin resistance gene from *Tn903* [17]. To construct pLF6, we used the pBluescript SK^+ fragment (Stratagene) carrying the *lacZ*α gene, which allowed the selection of recombinants on media with 3-bromo-4-chloro-5-indolyl β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactoside (IPTG). The variants of plasmids that carried fragments of the *mob* locus of pSUP5011 [18] were capable of mobilization with the aid of the helper plasmid RP4 belonging to the IncPα incompatibility group. *E. coli* strains TG1, C600, and HB101 and a number of gram-positive bacilli, lactobacilli, and enterococci served as recipients of the pLF1311 derivatives. Conjugative mobilization was carried out using the RP4-bearing strain C600 (C600*recA*::*Tn10*) as a donor.

E. coli strains and bacilli were cultivated in LB broth at 37°C under intense aeration. Enterococci and lactococci were cultivated in liquid M17 medium without aeration at 37 and 30°C, respectively. Lactobacilli were grown in MRS medium (Difco). Selective growth conditions were created by adding 50 μg/ml kanamycin or 15 μg/ml chloramphenicol (except the cultivation of recombinant *E. coli* strains in liquid media, when chloramphenicol was added to give a concentration of 5 μg/ml).

Conjugative mobilization. Plasmid DNA was transferred to cells through conjugative mobilization on 0.45-μm-pore-size nitrocellulose membrane filters (Millipore) [5]. Overnight donor and recipient cultures were mixed in a ratio of 10 : 1 and filtered through the aforementioned membranes. The membranes with

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adsorbed cells were incubated in petri dishes with a complete medium for 18–24 h. Cells were then washed from the filters with sterile physiological saline solution and plated on selective media to select ex-conjugants. The growth of the gram-negative donor was suppressed by adding polymyxin B or streptomycin to a concentration of 100 μg/ml.

DNA manipulations. Plasmid DNA was isolated through alkaline lysis. DNA restriction, molecular cloning, and analysis of recombinant clones were carried out by routine procedures [19].

RESULTS AND DISCUSSION

Since cryptic plasmids are difficult to manipulate, the cryptic plasmid pLF1311 was marked by the chloramphenicol acetyltransferase (*cat*) gene of plasmid pC194 from *Staphylococcus aureus* (positions 973 to 2008 according to the data of Horinouchi and Weisblum [16]). The *cat* gene was cloned at the *Pst*I site of pLF1311 by means of linkers. The resultant plasmid pLF2 was able to transform *Bacillus subtilis* and *E. coli* cells and made them resistant to chloramphenicol.

The functional regions of pLF1311 found by the method of deletion and insertion mapping with the subsequent analysis of their primary structure showed that pLF1311 can be referred to pE194-like plasmids, which replicate according to the rolling circle model [20, 21]. Plasmid pLF1311 is very similar to plasmid pLB4 from *Lactobacillus plantarum* [22]. The minimal region of pLF1311 necessary for its replication includes two open reading frames (ORFs) under a common promoter and the region of replication initiation (*ori*$^+$). The first downstream ORF (*repA*) codes for a 6-kDa polypeptide, which is highly homologous to the DNA-binding suppressors of the transcription of the pE194-like plasmids of gram-positive bacteria. The second ORF (*repB*) codes for a 27-kDa polypeptide, which is homologous to the protein that initiates σ-type replication by inserting a single-strand break into a special site. The respective *ori*$^+$ site was found within a minimum replicon as a region with the conservative DNA sequence representing a loop in the stem–loop structure possessing a high free energy of formation.

Outside the region of the minimum replicon, there is one more palindrome, which is less perfect but more extended (it comprises 40 bp). This palindrome was identified as a region of the preferential initiation of the replicative synthesis of the second DNA strand (*ori*$^-$). Such highly conservative *ori*$^-$ type sites have so far been revealed only in lactobacillar plasmids replicating according to the rolling circle model [6].

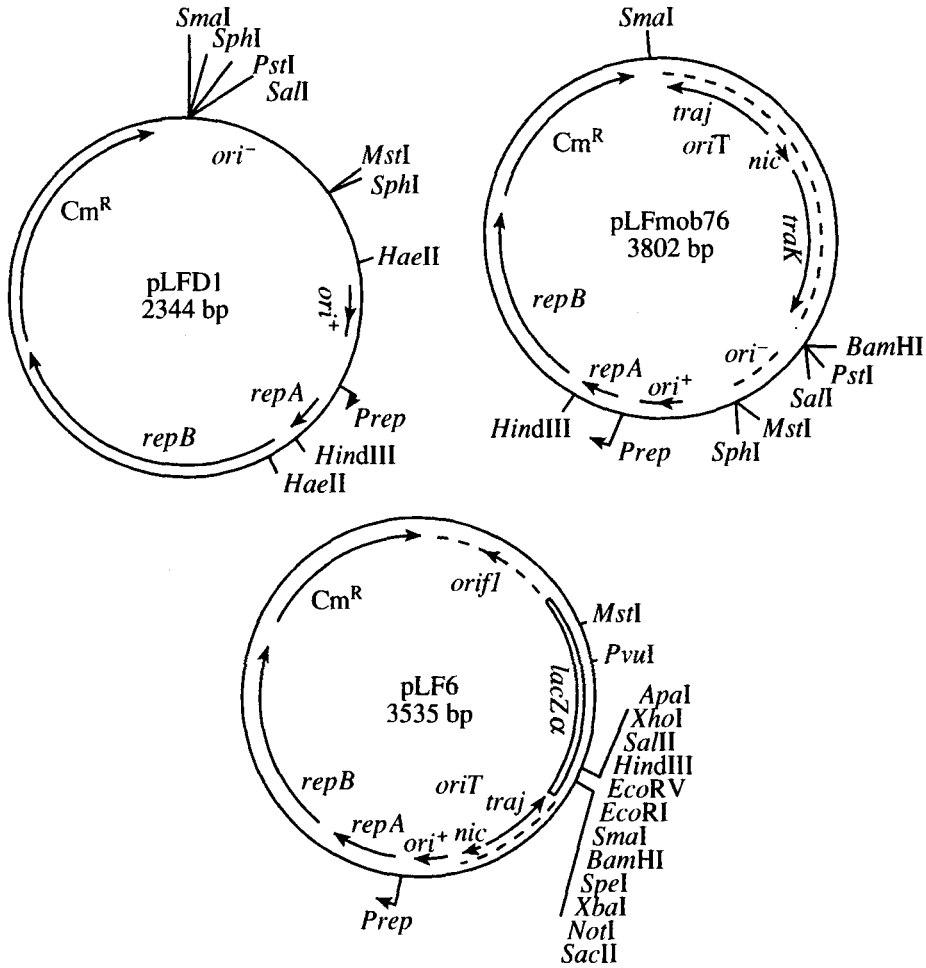
Based on these results, we constructed a family of cloning vectors lacking unknown DNA sequences.

The minimum vector of this family, pLFD1 (see figure), is a single-replicon shuttle vector of a small size (2344 bp), which permits DNA cloning at sites *Pst*I, *Sma*I, *Sal*I, *Sph*I, and *Mst*I. Vector pLFD1 retains the *ori*$^-$ locus, which accelerates the conversion of single-stranded DNA into double-stranded DNA during replication in an appropriate host.

Plasmid pLF-K1, constructed by the insertion of the *kan* gene from the *Tn903* into the *Pst*I site of pLFD1, allows the selection of recombinants through the insertion-induced inactivation of markers. This plasmid contains the following unique sites recognizable by restriction endonucleases: *Xho*I, *Pvu*I (within the *kan* gene), and *Nco*I (within the *cat* gene). The *kan* gene from *Tn903* provides for kanamycin resistance in a wide range of microorganisms, bacilli in particular, which allows either of these two markers to be used for inactivation. It should be noted that plasmids for lactobacilli, whose natural strains are usually resistant to kanamycin, must contain a genetic construction providing for chloramphenicol resistance as an active selective marker.

The most promising methods of DNA transfer to bacterial cells for which efficient transformation procedures are unknown are electrotransformation and conjugative mobilization from *E. coli* cells. For the mobilization of a nonconjugative plasmid to be efficient, it must contain the *cis*-region of initiation of conjugal transfer (*oriT*), whereas donor cells must contain the *trans*-products of the *tra* genes of the respective conjugative helper plasmid. The literature on the mobilization of double-replicon plasmids from *E. coli* to cells of *Corynebacterium glutamicum, Streptomyces* sp., *Mycobacterium smegmatis, Saccharomyces cerevisiae* and other species is now fairly extensive. Since replicon pLF1311 can be maintained in both gram-positive bacteria and *E. coli*, it was used to construct single-replicon mobilizable vectors. The mobilizable plasmid pLFVM2 was obtained by introducing the *Bam*HI fragment of plasmid pSUP5011 carrying the RP4-specific *oriT* site [18]. Another variant of this plasmid, designated as pLFRI, was obtained by inserting the *Eco*RI linker into the unique *Sma*I site of pLFVM2. The mobilizable plasmids pLFVM2 and pLFRI allow the cloning of genes in *E. coli* cells and then their conjugal transfer to various gram-positive bacteria.

We also constructed mobilizable plasmids of smaller sizes containing more restriction sites appropriate to cloning. Thus, plasmid pLFmob76 contains 3802 bp and the unique restriction sites *Bam*HI, *Pst*I, *Sal*I, *Sma*I, *Sph*I, and *Mst*I (see figure). Vector pLF6 (see figure) contains 3595 bp and carries the gene of the α-peptide of β-galactosidase from plasmid Bluescript SK$^+$ (Stratagene). Fifteen unique restriction sites of these vectors (*Apa*I, *Xho*I, *Sal*I, *Hin*dIII, *Eco*RV, *Eco*RI, *Pst*I, *Sma*I, *Bam*HI, *Spe*I, *Xba*I, *Not*I, *Sac*II, *Mst*I, and *Pvu*I) allow the selection of insertion into these sites by observing colored *E. coli* colonies grown on media with X-Gal and IPTG. The selected recombinant plasmids can be transferred to various gram-positive recipient bacteria from donors bearing the mobilizing plasmid RP4.



Vectors for lactic acid bacteria based on replicon pLF1311. *Ori*$^+$, *ori*$^-$, *repA*, and *repB* are the functional elements of replicon pLF1311; *Prep* is the promoter of the replicative genes of pLF1311; *Cm*^{R} is the chloramphenicol acetyltransferase (*cat*) gene of plasmid pE194 providing for chloramphenicol resistance; *oriT* is the region of initiation of the conjugal transfer of plasmid RP4 from the incompatibility group IncPα; *nic* is the nick site; *traJ* and *traK* are the genes of plasmid RP4 involved in conjugal transfer; *lacZα* is the gene encoding the α-peptide of the β-galactosidase of *E. coli*; *orif1* is the region of initiation of the replication of the filamentous bacteriophage f1.

Plasmid pLF6 carries the promoters of bacteriophages T3 and T7 flanking a polylinker (this enables an efficient in vivo transcription of cloned sequences) and the region of initiation of replication of the filamentous bacteriophage f1 from pBluescript SK$^+$ (this enables the obtaining of single-stranded DNA in *E. coli* cells with the aid of a helper bacteriophage, e.g., M17K07). All this makes vector pLF6 suitable for gene engineering manipulations with *E. coli*. The fact that pLF6 replicates according to the rolling circle model does not considerably hinder manipulations with this vector, as it takes place with other universal vectors, e.g., vectors based on the ColE1 replicon. During the replication of vector pLF6, intermediate single-stranded DNA should be allowed to convert into double-stranded DNA, which takes some time. As a result, a sufficient yield of plasmid DNA in *E. coli*, a natural host of plasmid pLF1311, can be achieved only after 18–24 h of cultivation (i.e., in the late stationary growth phase), whereas this is typically achieved after 6–12 h of cultivation. Another difficulty with plasmid pLF6 is associated with a low efficiency of expression of the inducible chloramphenicol resistance marker in *E. coli*. Chloramphenicol overdosage may substantially reduce the yield of plasmid DNA, although the yield of biomass will be sufficient. To avoid this, the concentration of chloramphenicol should not be higher than 15 μg/ml in agar media (in this case, specific color around the recombinant *E. coli* colonies grown on agar media with X-Gal and IPTG develops after 16–22 h of cultivation) and 5 μg/ml in liquid media (to attain a sufficient yield of plasmid DNA in this case, the plasmid-bearing strains of *E. coli* should be grown for 18–24 h).

The range of possible hosts of replicon pLF1311 was determined using its mobilizable derivatives. Like some other plasmids from gram-positive bacteria (pC194, pMV158, pSH71, pWV01), pLF1311 exhibits a broad range of possible hosts. The derivatives of this

Mobilization frequency and segregation stability of replicon pLF1311 in some bacteria

Recipient strain	Mobilization frequency	Segregation stability, %
Bacillus amyloliquefaciens B 1796	2×10^{-6}	98
B. subtilis B 1727 Sm^R	5×10^{-5}	96
B. thuringiensis subsp. *galleriae* B 1164	2×10^{-3}	96
B. thuringiensis subsp. *finitimus* B 1163	5×10^{-5}	ND
B. thuringiensis subsp. *kurstaki* B 3847	5×10^{-6}	56
Brevibacterium flavum B 120	5×10^{-8}	4
Escherichia coli HB101	1×10^{-2}	26
Enterococcus faecalis OG1	1×10^{-5}	100
E. faecium M 74	5×10^{-7}	100
Erwinia sp.	ND	ND
Lactobacillus acidophilus	ND	90
L. brevis VKM1303	3×10^{-8}	100
L. buchneri VKM 1310	1×10^{-7}	100
L. fermentum 90Tc4	1×10^{-6}	60
L. plantarum 8 RAZ	2×10^{-7}	90
Lactococcus lactis LPIT6	3×10^{-7}	100

Note: Mobilization frequency was calculated per one cell. Segregation stability was assessed after 40 generations. ND stands for "no data."

plasmid were introduced into many gram-positive bacteria and the gram-negative bacteria *E. coli* HB101 and *Erwinia* sp. (see table), but failed to be introduced into cells of *Lactobacillus acidophilus* 6a, *L. amylophilus* BVT 24/88, *L. bulgaricus* B2179, *L. casei* VKM 535, *Leuconostoc mesenteroides* ATCC 10830, *Pediococcus* sp. CCM 3770, *Streptococcus thermophilus* 58, *Micrococcus* sp., *Staphylococcus aureus* 3A, *Bacillus coagulans* 733, *B. licheniformis* 7, *B. stearothermophilus* 24, *Thermoactinomyces vulgaris* 936, or *Corynebacterium glutamicum* 95.

The efficiency of conjugative mobilization depends on many factors, including the degree of incompatibility of the introduced and resident plasmids. Thus, we failed to introduce the derivatives of pLF1311 into *L. fermentum* VKM 1311 cells containing the native cryptic plasmid pLF1311. The effect of another important factor, the activity of the restriction endonucleases of recipient cells, was studied in the case of plasmid mobilization in *Bacillus amyloliquefaciens*, a producer of the restriction endonuclease *Bam*HI. In this case, the mobilization frequency proved to be dependent on the number of *Bam*HI sites in donor cells and the degree of their in vivo methylation. The more than 1000-fold difference in the frequencies of plasmid mobilization in *B. thuringiensis* subsp. *galleriae* and subsp. *kurstaki* can be explained by the effect of the restriction enzymes. Since the failure of mobilization can also be related to nonoptimal conditions used for the conjugation of some recipients, we cannot exclude the possibility that the range of the hosts of pLF1311 derivatives is wider than is believed.

The ability of plasmids of the pLF1311 family to replicate in a wide range of gram-positive and gram-negative bacteria can be explained in terms of a specific structural organization of pLF1311. Indeed, the replication of this plasmid is initiated by its natural protein RepB encoded by the plasmid genome. Therefore, for replicon pLF1311 to be maintained in a wide range of hosts, it is necessary that the promoter of its *repB* gene is recognizable by the host RNA polymerases. This evidently takes place, since the pLF1311 promoter is very close to the consensus sequence.

To elucidate the possibility of gene cloning by pLF1311 derivatives, we attempted to clone the neutral proteinase gene of *Bacillus brevis* with the aid of vector pLF6 and plasmid pTM261 harboring this gene [23]. Chloramphenicol-resistant *E. coli* transformants produced white colonies when grown on plates with this antibiotic, IPTG, and X-Gal and were able to hydrolyze casein, as is evident from the formation (on the 3rd or 4th day of incubation) of clearing zones on plates with skim milk and chloramphenicol. Using conjugative mobilization, we introduced recombinant plasmids with the neutral proteinase gene into cells of *L. buchneri* VKM 1310, *E. faecalis* OG1, *B. subtilis* 168 Sm^R, and *B. subtilis* A773. We believe that further efforts to clone proteinase genes by means of vectors carrying the pLF1311 replicon may enhance the proteolytic activity of recipient strains, increase their ability to digest protein substrates, and, hence, improve their efficiency as probiotics or starters.

To conclude, we constructed a family of shuttle vectors based on the cryptic plasmid pLF1311 from *L. fermentum*. Some of these vectors possess valuable properties appropriate to gene engineering manipulations with DNA. In particular, they have small sizes (vector pLFD1), contain a selective marker of resistance to chloramphenicol or kanamycin (vector pLF-K1), can be transferred from *E. coli* into gram-positive bacteria (vectors pLFVM2, pLFR1, pLFmob76, pLF6), contain restriction sites suitable for cloning, and provide for the direct selection of *E. coli* recombinants based on the color of their colonies (plasmid pLF6). This family of vectors allows various genes to be cloned in lactobacilli, *E. coli*, and many gram-positive bacteria under their natural promoters.

ACKNOWLEDGMENTS

We are grateful to Yu.V. Yomantas for the construction of plasmid pLF2 and to V.G. Doroshenko, S.G. Karpushina, and S. Gronskii for help with some experiments.

This work was supported by the Russian Foundation for Basic Research, project no. 96-04-49239.

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