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EXPERIMENTAL ARTICLES

Expression Vector pLF22 for Lactic Acid Bacteria

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Abstract—The construction of the expression vector pLF22 for lactic acid bacteria is described. The vector contains a replicon of the cryptic plasmid pLF1311 from *Lactobacillus fermentum* and a multiple cloning site of the *lacZ'* gene integrated with the plasmid *rep* operon. Such a construction of the vector provides for the constitutive transcription of the cloned sequences lacking the terminators of transcription in all the strains that maintain the replication of the vector. The vector is suitable for a wide range of gram-positive and gram-negative bacteria, including probiotic strains. The efficiency of the vector was verified by expressing the β-galactosidase gene in a laboratory *Escherichia coli* strain and the synthetic gene of growth hormone-releasing factor (GRF) in the probiotic strains of lactobacilli and enterococci. A recombinant strain with the GRF gene included in the diet of laboratory animals exerted affected characteristic features of their physiology, anatomy, and growth.

Key words: plasmid DNA, gram-positive bacteria, probiotics, somatoliberin.

Lactic acid bacteria (lactobacilli, lactococci, and enterococci) are a vast group of gram-positive bacteria. Numerous vector systems were successfully engineered for gene expression in these biotechnologically important microorganisms [1–6], but their efficiency is lower than that of bacillar or enterobacterial vectors. The transcription of foreign genes in lactobacilli was accomplished from native [5] and artificial [4] promoters. The incorporation of foreign sequences into a replicative operon of a plasmid does not necessarily affect replication [7, 8].

The aim of this work was to construct a vector that would provide for the transcription of cloned sequences in a wide range of lactic acid and other bacteria from the promoters of replicative genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids used in this study were derivatives of the cryptic plasmid pLF1311 from *Lactobacillus fermentum* VKM 1311 [3]; vectors pUK21 and pTZ19R; plasmid pGRF1 [9]; plasmid pJEL24 bearing the *lacZ* gene without the initiating codon (obtained from A.S. Mironov, Institute for Genetics and Selection of Industrial Microorganisms); and plasmid pUKTZ, a modified version of pUK21 obtained by replacing a small *Pvu*II fragment with the respective fragment of plasmid pTZ19R.

Selective agar media contained 15 μg/ml chloramphenicol (Sigma). Plasmid-bearing *E. coli* strains were grown in a liquid medium containing 5 μg/ml chloramphenicol. DNA was isolated from cells grown to the late stationary phase (18–24 h of cultivation) [3]. Gram-positive recipients were the nonpathogenic enteric lactic acid bacteria *Lactobacillus acidophilus* K3, *Lactobacillus* sp. 8RAZ, *Enterococcus faecalis* OG1 from the laboratory collection, and *Enterococcus faecium* M74 (a component of the probiotic preparation Enteracid P).

Polymerase chain reaction (PCR). The somatoliberin sequence was amplified and fused with the initiation codon of translation by using oligonucleotide primers MI and MII. The MI primer d(GAAGGATAAATTTATGTACGATATCT) contained a potential SD region, an initiating codon, and an AT-rich spacer between them. The MII primer had the structure d(TCCATATTGGTCGACTATTAA). PCR amplifications (25 cycles) were run at 93°C for 1 min, at 50°C for 1 min, and at 72°C for 1 min. The products were purified by electrophoresis in 4% agarose, phosphorylated, and cloned into the *Eco*RV site of pBluescriptKS$^+$ vector (Stratagene).

DNA cloning. Molecular cloning and the analysis of recombinant *E. coli* clones were performed by conventional techniques using restriction endonucleases, the DNA ligase of phage T4, and other relevant enzymes purchased from SibEnzyme (Novosibirsk, Russia) and MBI Fermentas (Lithuania).

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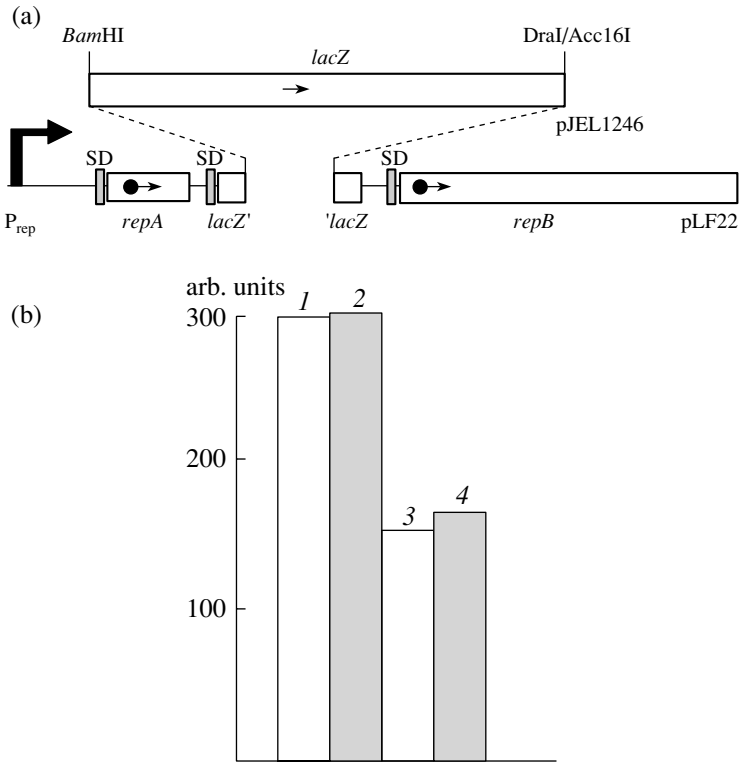


Fig. 1. (a) The structure of the *lacZ* gene cloned on plasmid pLF23 and (b) its expression in the recombinant cells grown to (*1, 2*) the early and (*3, 4*) the middle exponential phase (*2, 4*) in the presence and (*1, 3*) in the absence of IPTG in the medium. The expression of the *lacZ* gene was estimated in vitro by the activity of β-galactosidase in arbitrary units [10].

β-Galactosidase was assayed colorimetrically with *o*-nitrophenol-β-D-galactoside as the substrate [10].

Keeping of laboratory animals. Experiments were carried out with three groups of hybrid rabbits (Californian chinchilla × Soviet chinchilla), each containing three males and four females. Young rabbits were kept in mesh cages (one or two animals per cage). The rabbits of the control group were given a basic diet. The rabbits of the first experimental group were fed on a diet enriched in the recipient strain (about 4×10^9 cells per day). The rabbits of the second experimental group were on a diet enriched in the recombinant strain (about 4×10^9 cells per day). The experiments lasted from 2 to 4 months.

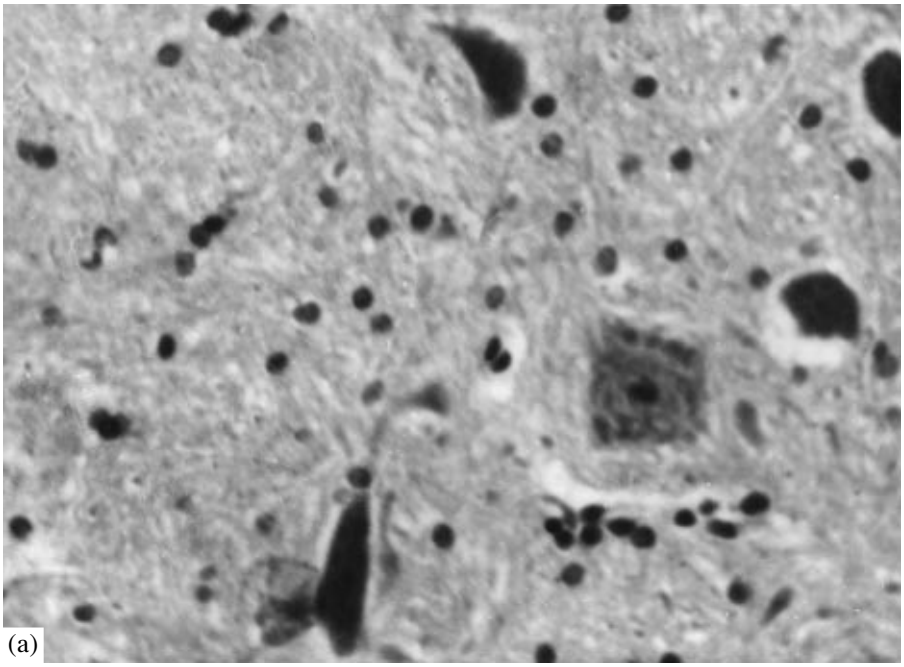
Histological and electron microscopic studies were carried out as described elsewhere [11]. The supraoptic core of the hypothalamus was examined on serial sections. The functional state of neurocytes was estimated by the amount of homori-positive material. The area of the neuron nuclei and bodies on images was measured with an eyepiece micrometer. The mitotic coefficient for the anterior pituitary was defined as the number of mitotic divisions per 500 cells. Thin sections were examined with an EM100K electron microscope.

The results obtained were statistically processed by using Student's *t*-test.

RESULTS AND DISCUSSION

The incorporation of *lacZ*α into the *repAB* operon. Vectors based on the pLF1311 replicon [3] retain the structural organization of the natural plasmid, which belongs to the family of pE194-like plasmids. The replication of these vectors depends on the expression of the *rep* operon genes, the first of which codes for the RepA protein (a negative transcriptional regulator), and the second of which codes for the RepB protein, which initiates σ-type replication from the *ori*$^+$ site of plasmid DNA [12]. These genes are transcribed from a common promoter. This promoter is functional in all the strains which maintain plasmid, since the RepB protein is strictly necessary for plasmid replication. The incorporation of an additional nucleotide sequence into the operon must thus provide for its transcription in all potential hosts of the pLF1311 replicon [3].

The *repA* and *repB* genes of plasmid pLF1311 are separated by a 76-bp spacer with the *Hin*dIII and *Hae*II restriction sites. The construction procedure for the expression vector included several steps, which gave rise to plasmid pLF22. The respective spacer in this plasmid was replaced (after blunting the *Hin*dIII sticky end) by the *Bsr*BI–*Hae*II region of plasmid pUKTZ carrying promoter-depleted *lacZ*α gene, in the same orientation as the replicative operon genes. Since the *Bsr*BI site is located within the operator of the *lac*



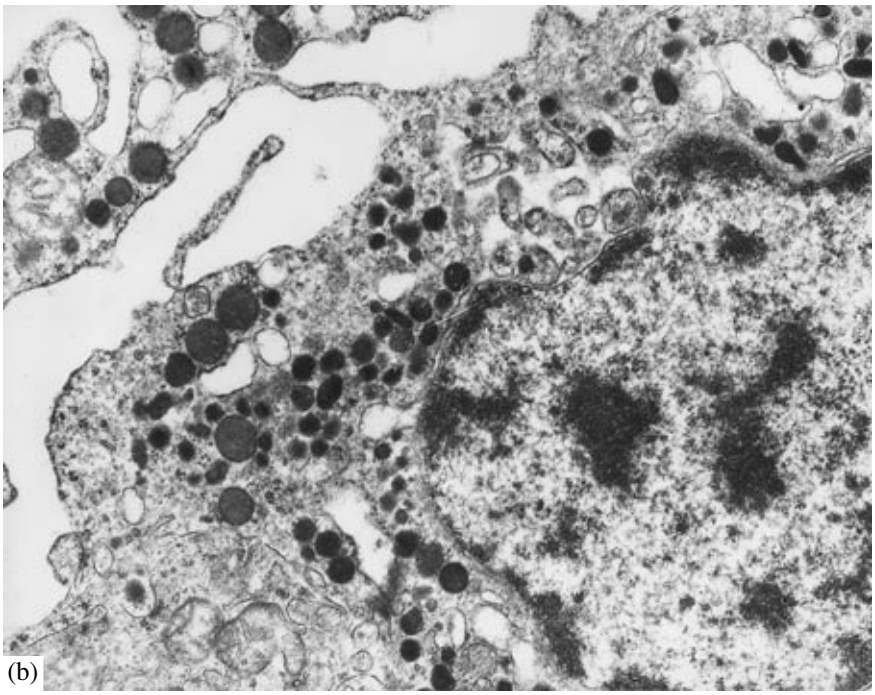


Fig. 2. The structure of the hypothalamus and the anterior pituitary of an experimental rabbit that received exogenous somatoliberin. (a) Homori-stained hypothalamus at a magnification of 400×: easily visible are large, light neurons with a vesicular nucleus surrounded by a few small granules. (b) Adenohypophysis at a magnification of 20000×: easily visible are hypertrophied nucleoli, which are diffuse due to the elevated content of filamentous material. (c) Adenohypophysis at a magnification of 10000× with the activated processes of synthesis and secretion in somatotropocytes.

operon, the cloned fragment *lacZ*α is not subject to the action of the *lac* repressor. The expression of *lacZ*α provides for complementation in *E. coli* TG1(pLF22), due to which the colonies of this strain grown on agar plates with chloramphenicol and Xgal are blue in color, irrespective of whether IPTG (the inducer of the *lac* operon) is present in the medium or not.

The expression of the pLF22-cloned gene in *E. coli*. The blue color of the *E. coli* TG1(pLF22) colonies grown on agar plates with Xgal indicated the postulated transcription of the *repA* + *lacZ*α + *repB* mRNA from the promoter of the replicative plasmid genes and the synthesis of α-peptide. To estimate the efficiency of the replicative promoter for expressing nucleotide

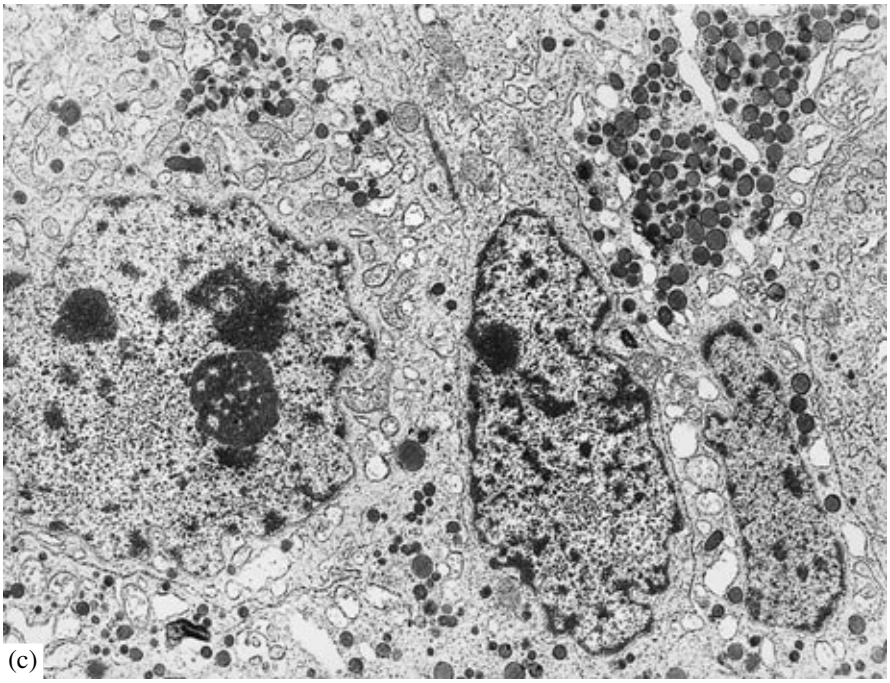


Fig. 2. (Contd.)

sequences in *E. coli* strains, we cloned the coding region of the β-galactosidase gene, which was derived from plasmid pJEL24 as the full-length ORF of *lacZ* without the starting codon and ligated with the DNA of vector pLF22 hydrolyzed by *Bam*HI and *Acc*16I. The resulting plasmid pLF23 encoded the fused β-galactosidase protein (Fig. 1a) and provided for its high expression in Lac^- strains. The expression of this protein was estimated by the activity of β-galactosidase with *o*-nitrophenyl-β-galactopyranoside, which did not depend on the presence of the *lac* operon inducer IPTG in the medium (Fig. 1b). In this case, the β-galactosidase activity comprised about one-third of the β-galactosidase activity of Lac^+ strains with the full activation of the *lac* promoter [10]. The expression of the protein was especially high in the exponential-phase culture, when the plasmid replicative genes probably have the maximum activity. In the stationary-phase culture, when plasmids are replicated slowly, the activity of β-galactosidase declined (Fig. 1b).

Expression in recipient lactic acid bacteria. The expression of the cloned sequences in gram-positive recipients was studied by investigating the effect of the recombinant strains with the cloned eukaryotic gene of somatoliberin on laboratory animals (rabbits). Somatoliberin (or the growth hormone–releasing factor) is a peptide composed of 44 amino acid residues, which is synthesized in the hypothalamus of vertebrate animals and acts on the hypophysis cells, causing them to synthesize the growth hormone (somatotropin) [13]. The use of peptide hormones in livestock raising is a good alternative to feeding corticosteroid hormones, which, unlike ecologically safe peptide hormones, accumulate in animal tissues and organs and then in the environment. In addition, bacterial cells expressing peptide hormones can be conveniently administered perorally as affordable crude preparations (see below), whereas usually peptide hormones are administered by injection in the form of expensive highly purified preparations.

To express somatoliberin in bacterial cells, the synthetic gene sequence derived from plasmid pGRF1 [9] was incorporated into an expression vector. The 3'-end of the amplified primer MI was complementary to the coding region of the somatoliberin gene in pGRF1, and its 5'-end contained the SD sequence with the AT-rich spacer and the initiating codon of mRNA translation in bacterial cells. The amplification product was first cloned in *E. coli* on the pBluescriptKS$^+$ vector, and then its fragment with the proper orientation of the somatoliberin ORF was cloned on vector pLF. The derived plasmid pLF-SL was introduced into recipient probiotic strains. The action of the resulting recombinant strains on laboratory animals was compared with the effect of the recipient strains.

The physiological responses of animals to exogenous somatotropin and somatoliberin are well known [14–16]. Peroral administration of recombinant strains is likely to be effective due to the peptide's ability to safely pass through the intestinal barrier, as has been repeatedly shown in experiments with animals and isolated tissues [17, 18]. As is evident from the table, the laboratory rabbits responded to the administration of three out of the four recombinant strains used in the experiments with an increase in growth rate, the linear

The effect of the recombinant strains carrying the somatoliberin gene under the promoter of the replicative genes of the vector on the relevant parameters of laboratory rabbits

Parameter	Strain added to basic diet			
	Lactobacillus acidophilus K3 (pLF-SL)/recipient[a]	*Lactobacillus* sp. 8RAZ (pLF-SL)/recipient[a]	*Enterococcus faecium* M74 (pLF-SL)/recipient[b]	*Enterococcus faecalis* OG1 (pLF-SL)/recipient[b]
Average live mass of young rabbits, g	$\frac{2470 \pm 34}{2330 \pm 19}$	$\frac{2720 \pm 48}{2690 \pm 56}$	$\frac{3880 \pm 64}{3686 \pm 30}$	$\frac{3133 \pm 98}{3140 \pm 114}$
Percent weight gain relative to the control	$\frac{111*}{101}$	$\frac{120}{119}$	$\frac{119}{114}$	$\frac{104}{105}$
Fat content in half-carcass, %	$\frac{5.7*}{7.7}$	$\frac{4.8*^{c}}{8.5^{c}}$	$\frac{2.4*}{3.2}$	$\frac{5.8}{7.3}$
Diameter of adipocytes in adipose tissue, μm	$\frac{70 \pm 4*}{102 \pm 3}$	$\frac{71 \pm 4*}{112 \pm 4}$	ND	ND
The mitotic coefficient of adenohypophysis cells	$\frac{3.2 \pm 0.3*^{c}}{0.8 \pm 0.1^{c}}$	$\frac{3.1 \pm 0.1*^{c}}{0.8 \pm 0.1^{c}}$	ND	ND

Note: ND stands for "not determined." [a], [b], and [c] refer to the experiment duration of 3, 4, and 2 months, respectively.

* The asterisks mark the data with statistically significant differences between the control and the experimental values estimated for $P < 0.05$.

size of bodies, and mitotic activity in some tissues and a decrease in the fat content of tissues and the diameter of adipocytes in adipose tissue. The changes in these parameters were statistically significant (table).

It is known that somatoliberin acts on the cells of the anterior pituitary. A comparative histological analysis of the hypothalamuses and the hypophyses of the control and experimental (fed on the recombinant strain–containing diet) rabbits revealed enhanced functional activity of the supraoptic core neurons of the hypothalamus of the experimental rabbits, as is evident from the predominance of large, light neurons with a vesicular nucleus surrounded by few small granules arranged as a narrow band (Fig. 2a). The area of the neuron nuclei in the experimental rabbits was greater than in the control rabbits. The hypophysis of the experimental rabbits contained 5% more somatotropocytes than did the hypophysis of the control rabbits. The proliferative activity of the adenohypophysis cells is known to be stimulated by the peptides of the supraoptic core [19, 20]. Since the anterior pituitary has a low rate of cell proliferation (on the norm), the difference in the mitotic activity of the anterior pituitary cells of the control and experimental rabbits should be considered significant (table). The activation of the anterior pituitary cells was also manifested in a marked hypertrophy of nuclei and particularly nucleoli, which became diffuse due to the elevated content of filamentous material (Fig. 2b). The synthesis and secretion of secretory granules in the somatotropocytes of the experimental rabbits were more intense than in the somatotropocytes of the control rabbits (Fig. 2c).

All these data indicate enhanced proliferative and secretory activities in the adenohypophyses of the rabbits fed with the recombinant lactic acid bacteria carrying the expressed somatoliberin gene. Such a response of the laboratory animals is similar to the response of the animals that received exogenous somatoliberin or somatotropin by injection or implantation and suggests that the expression of somatoliberin from the replicative gene promoter of the cloned vector is sufficiently high to induce notable physiological effects. The less pronounced effect of the recombinant strain *Enterococcus faecalis* OG1(pLF-SL) can be explained by a lower segregation stability of pLF plasmids in this strain, a lower efficiency of the pLF-SL promoter or the synthetic SD sequence, a more active proteolysis of foreign peptides, a weaker adhesion of *E. faecalis* OG1 cells to the intestinal epithelium, or other factors, whose study was not the aim of this work. It should be noted that the diminished expression of somatoliberin in the *E. faecalis* OG1 cells indicates that the developed vector systems can be used for a rapid search for promising recipient strains. The notable physiological effect of the recombinant bacteria occurring in the gastrointestinal tract can be related to the fact that the amount of the recombinant hormone that was released from bacterial cells and passed unchanged through the intestinal wall and the blood–brain barriers was sufficiently large to activate the target cells of the anterior pituitary.

To conclude, we developed a vector system for the heterologous expression of prokaryotic, eukaryotic, and artificial genes in gram-positive and gram-negative bacteria and varified its efficiency with the soma-

toliberin gene. This system can be used for a rapid search for promising recipient strains.

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