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APPLICATION OF THE VECTOR pEC-XK99E FOR THE CONSTRUCTION OF RECOMBINANT STRAIN-PRODUCERS OF L-ARGININE BASED ON *BREVIBACTERIUM FLAVUM*

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Using *Escherichia coli – Corynebacterium glutamicum* shuttle expression vector pEC-XK99E, molecular cloning of the heterologous gene *argJ* of the thermophilic bacterium *Geobacillus stearothermophilus*, as well as of homologous genes *argG* and *argH* of *Corynebacterium glutamicum* was carried out in cells of coryneform bacterium *Brevibacterium flavum*. It was shown that expression of cloned *arg* genes from the strong promoter *Ptrc* (its activity is controlled by the protein repressor $LacI^q$) occurred regardless of the presence of transcription inducers lactose or IPTG in the medium.

***Keywords*:** molecular cloning, vector pEC-XK99E, *arg* genes, *Brevibacterium flavum*, strong promoter *Ptrc*, lactose, IPTG.

Introduction. The rapid development of the methodology of genetic engineering made it possible to create producers of many biologically active compounds, including amino acids, in a relatively short time.

The increased need for amino acids and their derivatives, associated with the appearance of new areas of their application and the extension of old ones, required further improvement of producers both in terms of increasing their activity and improving other technological indicators of the strains. This problem can be solved in its most complete form using the methodology of genetic engineering, which allows to increase the expression of the desired gene by multiplying it on multi-copy vectors. For a long time genetic engineering methods have not been widely used to create corynebacterial strain-producers, although non-pathogenic coryne-form bacteria have long established themselves in large-scale production of amino acids from the best side. This was due to the lack of a "host-vector" system for molecular cloning of genes in corynebacteria.

The identification of endogenous plasmids was a prerequisite for the development of recombinant DNA technologies for the creation of amino acid producers based on coryneform bacteria [1, 2].

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The most widely used are shuttle vectors, viz. bireplicon plasmids that can replicate in cells of various kinds of bacteria. Shuttle vectors, replicating in *Corynebacterium glutamicum* and *Escherichia coli* (or *Bacillus subtilis*) cells, were derived based on endogenous plasmids of *C. glutamicum* and plasmids replicating in *E. coli* (or *B. subtilis*) [3].

The goal of this work was to use the *E. coli–C. glutamicum* shuttle expression vectors pEC-XK99E for molecular cloning of *arg* genes in *Brevibacterium flavum* cells, as well as the construction of strain-producers of L-arginine.

Materials and Methods. The following strains were used in this work: *Br. flavum* HK-19A (ile$^-$, D-ser^s, ArgHx^r, TA^r), *C. glutamicum* ATCC 13032, *Geobacillus stearothermophilus* NCIB 8224, *E. coli* XA4 (F$^-$ *argA nalA* $\lambda^-\lambda^s$ *trpR hsdR*), *E. coli* XS1D2R (F$^-\Delta$*(ppc–argE)* 101 *nalA rpoB* λ^- *hsdR recA*), *E. coli* XG31 (F$^-$ *argG his rpoB rpsL* $\lambda^-\lambda^s$ *hsdR*), *E. coli* XH11 (F$^-$ Δ(*ppc–argECBH*) MN42 *nalA rpoB* $\lambda^-\lambda^s$ *hsdR*). For the cultivation of the strains of *Br. flavum* and *C. glutamicum*, meat-peptone broth (MPB) and meat-peptone agar (MPA) were used. For growing strains of *E. coli* and *G. stearothermophilus*, LB-broth and LB-agar were used as nutrient media [4], and for *E. coli* strains, liquid and agar M9 with necessary additions were used as synthetic media [4]. L-amino acids at the concentration of 40 *mg*/*mL* were added to the medium M9. To cultivate the recombinant strains, kanamycin (Km) was added to the media at a concentration of 50 $\mu g/mL$.

Isolation of plasmid DNA was performed according to the "QIAGEN" (Germany) protocol using the QIAprep Spin Miniprep Kit. Isolation of chromosomal DNA of *G. stearothermophilus* was carried out according to the described procedure [5], and the same for *C. glutamicum*, as described in [6].

DNA analysis was performed using 1% agarose gel prepared in Tris-acetate buffer [6], under the UV of the "FOTODYNE Inc" device (USA). Polymerase chain reaction (PCR) was carried out using "Gene Cycler" ("Bio-Rad", USA) according to the "Invitrogen" (USA) protocol. The temperature cycling protocol was set as follows: initial denaturation for 5 *min* at 94°*C*; 30 cycles of: denaturation (30 *s* at 94°*C*), "annealing" (30 *s* at 52°*C*) and synthesis (2 *min* at 72°*C*); final synthesis (7 *min* at 72°*C*). DNA amplification was confirmed by electrophoresis.

Isolation and purification of PCR fragments obtained from the agarose gel was carried out using the QIAquick Gel Extraction Kit according to the "QIAGEN" protocol. The restriction was carried out, following the protocol specified by "New England Biolabs" (USA). Restriction endonucleases *Eco*RI, *Bam*HI and *Xba*I were used. Ligation was performed using the Quick Ligation^{TM} Kit, according to the protocol of "New England Biolabs".

Transformation of *E. coli* cells was carried out according to the known method [6]. Transformation (electroporation) of *Br. flavum* cells was performed according to the method described in [7], carrying out some modifications.

To carry out the flask fermentation, separate colonies of *Br. flavum* were transferred to MPB and grown in test tubes for 18 *h* at 30°*C*. Fermentation was carried out in 500 *mL* Erlenmeyer flasks with 14 *mL* of fermentation medium and 1 *mL* of seed material on a shaker at 220–240 *rpm* at 31°*C* for 72 *h*.

To determine the arginine-producing ability, the fermentation synthetic medium (medium C) of the following composition was used, %: sucrose – 15.0; $(NH_4)_2SO_4$ – 5.5, KH_2PO_4 – 0.3, $MgSO_4\times7H_2O$ – 0.1, $FeSO_4\times7H_2O$ – 0.001,

$MnSO_4 \times 5H_2O$ – 0.001, $CaCO_3$ – 5.0; biotin – 500 $\mu g/L$; thiamine – 500 $\mu g/L$; L-isoleucine – 300 $\mu g/mL$; pH of the medium – 7.6 [8].

Quantitative determination of L-arginine in the culture liquid was performed colorimetrically using the modified Sakaguchi method based on the specific staining of diacetyl with the guanidine group of arginine in the presence of 8-hydroxyquinoline in an alkaline medium [9]. Quantitative determination of L-arginine and related amino acids was also carried out using an amino acid analyzer "Shimadzu Nexera X2" (Japan).

Results and Discussion. In order to construct recombinant strain-producers of L-arginine, the resulting strain *Br. flavum* HK-19A (ile$^-$, D-ser^s, ArgHx^r, TA^r) was used as a recipient [10]. To create new arginine-synthesizing recombinant strains of coryneform bacteria, we carried out molecular cloning of the heterologous gene *argJ* of *G. stearothermophilus*, as well as of homologous genes *argG* and *argH* of *C. glutamicum* in the cells of the recipient strain *Br. flavum* HK-19A.

The chromosomal DNAs of bacteria *G. stearothermophilus* NCIB 8224 (gene *argJ*) and *C. glutamicum* (genes *argG* and *argH*), which served as a matrix for amplification of the corresponding genes, were isolated. Amplification of these genes was performed by PCR. For molecular cloning of these genes we have chosen *E. coli–C. glutamicum* shuttle expression vector pEC-HK99E (Fig. 1), which is based on medium copy plasmid pGA1 (4.9 *kb*) of *C. glutamicum*. The pGA1 plasmid contains the gene *repA*, the product of which is the protein of initiation of replication, and the gene *per*, which encodes a positive effector of replication that has a significant effect on the number of copies of the plasmid and on its segregational stability in *C. glutamicum* cells [11]. Vector pEC-XK99E (7.0 *kb*) contains a polylinker (mcs) with 13 sites for restriction endonucleases, as well as a strong promoter *Ptrc* of *E. coli*, regulated by the *lac*-operon repressor – LacI^q (the *lacI^q* gene product, which is in front of the promoter *Ptrc*) and induced in the presence of lactose or IPTG (isopropyl β-D-1-thiogalactoside). The vector also contains the Km-resistance gene – *aph*(3′)-*IIa* and transcription terminators T1 and T2 [12].

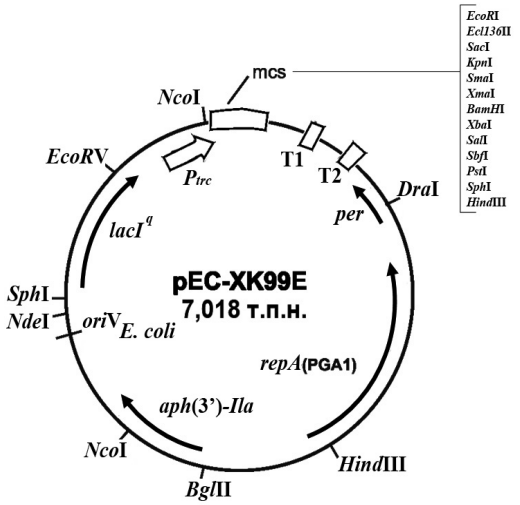

Fig. 1. Restriction map of the shuttle expression vector pEC-XK99E.

The obtained PCR products of the *argJ*, *argG* and *argH* genes were extracted from the gel and cloned into the vector pEC-XK99E (under the control of the promoter *Ptrc*), pretreated with restriction enzymes *Eco*RI/*Bam*HI (for the *argJ* and *argH* genes) and *Eco*RI/*Xba*I (for the *argG* gene). The competent cells of *E. coli* strains mutant for the genes *argA* (*E. coli* XA4) and *argE* (*E. coli* XS1D2R), since the product of *argJ* gene of *G. stearothermophilus* is a bifunctional enzyme, it complements two different mutations in the *E. coli* cells: $argA^-$ and $argE^-$, and *E. coli* strains mutant for the genes *argG* (*E. coli* XG31) and *argH* (*E. coli* XH11), respectively, were transformed by ligated mixture. The selection of transformants was carried out by complementation of the corresponding mutant genes on the synthetic medium M9 without arginine in the presence of Km (50 $\mu g/mL$).

The presence of cloned *arg* genes in the composition of constructed recombinant plasmids was confirmed by the results of complementation of arg^- mutations in the auxotrophic strains of *E. coli*, as well as by restriction analysis of these plasmids. Constructed recombinant plasmids pARGJ, pARGG, pARGH were used to create new arginine-producing strains based on *Br. flavum* HK-19A.

In order to create new recombinant strains of coryneform bacteria producing L-arginine, the constructed plasmids carrying the heterologous gene *argJ*, homologous genes *argG* and *argH*, and the vector pEC-XK99E (as a control) were transferred by electroporation into the recipient strain *Br. flavum* HK-19A obtained by us. The selection of transformants was performed on a complete medium in the presence of Km (50 $\mu g/mL$). After incubation at 30°*C* for 48 *h*, Km-resistant transformants of the *Br. flavum* HK-19A strain were selected from the plates.

Selected colonies were tested for arginine-producing ability in the conditions of flask fermentation in nutrient medium C (Fig. 2).

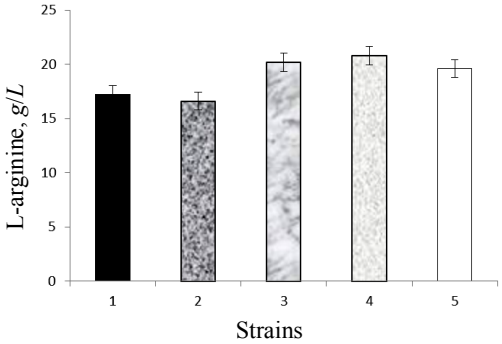

Fig. 2. Comparative arginine synthesizing activity of the strain *Br. flavum* HK-19A (1) and recombinant strains, carrying plasmids pEC-XK99E (2), pARGJ (3), pARGG(4) and pARGH(5).

As described above, the vector pEC-XK99E contains strong promoter *Ptrc* of *E. coli*, which is regulated by the protein-repressor $LacI^q$ and induced in the presence of lactose or IPTG. It has been found that the binding of RNA polymerase to the *lac*-promoter is relatively weak, and therefore the transcription of the *lac*-operon should be induced. In the absence of lactose in the medium, *lac*-promoter of *E. coli* is repressed. This means that it is turned off by a protein-repressor $LacI^q$, which blocks the transcription of the *lac*-operon. Induction or switching-on of the *lac*-operon occurs when lactose or IPTG is added to the medium. Both of these compounds prevent the binding of repressor to the *lac*-operator, and transcription is resumed [13].

In order to eliminate or reduce the repressive effect of the protein-repressor $LacI^q$ on the activity of the promoter *Ptrc*, lactose or IPTG in various concentrations were added into the fermentation medium (medium C). When adding lactose, the amount of sucrose also varied, so that the final concentration of carbohydrates in the fermentation medium was 15% (150 *g*/*L*). Added concentrations of lactose were made up as follows:

medium CL-1 – from 150 *g*/*L* of carbohydrates contained in the fermentation medium C, lactose was 12.5% and sucrose – 87.5%;
medium CL-2 – lactose 25.0%, sucrose – 75.0%;
medium CL-3 – lactose 50.0%, sucrose – 50.0%;
medium CL-4 – lactose 87.5%, sucrose – 12.5%.

IPTG was added in the fermentation media C at concentrations of 100 $\mu g/mL$ (CI-1 medium), 250 $\mu g/mL$(CI-2 medium) and 500 $\mu g/mL$ (CI-3 medium).

Fermentation in the presence of a specific concentration of lactose or IPTG was performed with the recipient strain *Br. flavum* HK-19A and recombinant strains containing the vector pEC-XK99E and recombinant plasmids pARGJ, pARGG, pARGH. The results of the flask fermentation are presented in Tabs. 1 and 2.

Table 1

Accumulation of L-arginine in fermentation medium containing different concentrations of lactose (relative to the level of L-arginine synthesized in the control fermentation medium (medium C))

Strains	Yield of L-arginine in presented fermentation media, %				
	C	CL-1	CL-2	CL-3	CL-4
Br. flavum HK-19A	100	115.1	103.4	100.2	56.8
Br. flavum HK-19A (pEC-XK99E)	100	105.2	102.3	98.2	68.1
Br. flavum HK-19A (pARGJ)	100	123.5	112.4	104.5	70.2
Br. flavum HK-19A (pARGG)	100	126.8	115.1	106.8	71.1
Br. flavum HK-19A (pARGH)	100	120.1	109.2	101.9	69.2

Table 2

Accumulation of L-arginine in fermentation medium containing different concentrations of IPTG (relative to the level of L-arginine synthesized in the control fermentation medium (medium C))

Strains	Yield of L-arginine in presented fermentation media, %			
	C	CI-1	CI-2	CI-3
Br. flavum HK-19A	100	102.8	103.7	102.3
Br. flavum HK-19A (pEC-XK99E)	100	101.6	102.1	85.2
Br. flavum HK-19A (pARGJ)	100	103.1	104.2	73.4
Br. flavum HK-19A (pARGG)	100	105.2	106.1	75.6
Br. flavum HK-19A (pARGH)	100	102.9	103.6	72.8

The results of experiments in this series showed that to reduce the repressive activity of the protein repressor $LacI^q$, it is advisable to use lactose at a concentration of 12.5% (medium CL-1) and IPTG – 250 $\mu g/mL$ (medium CI-2) as inducers. The level of synthesized L-arginine in the fermentation medium with the addition of lactose was significantly increased in all studied strains (both plasmid-free and plasmid-containing) compared to the level in the control medium. In the

case of the recipient strain *Br. flavum* HK-19A, as well as recombinant strain *Br. flavum* HK-19A, containing the vector pEC-XK99E, the increased synthesis of L-arginine may be the result of good utilization of lactose. As for recombinant strains containing the gene *argJ* of *G. stearothermophilus* and genes *argG*, *argH* of *C. glutamicum*, it can be concluded that, along with good utilization of lactose, induction of transcription of these genes from the strong promoter *Ptrc* in the presence of lactose also occurs. However, as can be seen from the results given in Tab. 1, a further increase in the concentration of lactose in the fermentation medium leads to inhibition of L-arginine biosynthesis. This is probably due to the accumulation of galactose, resulting from the splitting of lactose [14].

The production of L-arginine in the fermentation medium with the addition of IPTG was characterized by a slight increase in the yield of the final product (Tab. 2), which correlates with literature data, according to which the majority of corynebacterial strains has rather weak permeability of the cell wall relative to IPTG [15].

Conclusion. The results of this research show that the expression of cloned *arg* genes from the strong promoter *Ptrc* (its activity is controlled by the protein repressor $LacI^q$) occurs regardless of the presence of transcription inducers lactose or IPTG in the medium. This is evidenced not only by the results of complementation of arg^- mutations in auxotrophic strains of *E. coli*, but also by the relatively high synthesis of L-arginine in recombinant strains. This may be due to incomplete blocking of the transcription by the enterobacterial protein repressor $LacI^q$, which is consistent with the literature data regarding the weak repression of the lactose promoter in some corynebacterial strains, including *C. glutamicum* ATCC 13032 [15].

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