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SELECTIVE SYSTEM BASED ON FRAGMENTS OF THE M1 VIRUS FOR THE YEAST *SACCHAROMYCES CEREVISIAE* TRANSFORMATION

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❀ **Background.** A selective system based on the M1 virus of the yeast *Saccharomyces cerevisiae* was proposed. **Methods.** To create a recipient strain, a DNA fragment encoding the killer toxin of the M1 virus under the control of the regulated promoter of the *GAL1* gene was inserted into the genome of *S. cerevisiae* strains Y-1236 and Y-2177. **Results.** Integration of such expression cassette leads to the conditional lethality – resulting strains die on a medium with galactose when killer toxin synthesis occurs. A linear DNA fragment containing the gene of interest flanked by sequences homologous to the promoter of the *GAL1* gene and the termination region of the *CYC1* gene is used to transform the obtained strains. During transformation due to homologous recombination, the sequence encoding the killer toxin is cleaved and the transformants grow on a medium with galactose. **Conclusion.** The proposed selective system combines the main advantages of other systems: the use of simple media, without the need to add expensive antibiotics, and a simplified technique for constructing expression cassettes and selecting transformants.

❀ **Keywords:** yeast *Saccharomyces cerevisiae*; killer-toxins; M1 and M28 viruses; selective markers.

СЕЛЕКТИВНАЯ СИСТЕМА НА ОСНОВЕ ФРАГМЕНТОВ ВИРУСА M1 ДЛЯ ОТБОРА ТРАНСФОРМАНТОВ ДРОЖЖЕЙ *SACCHAROMYCES CEREVISIAE*

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❀ **Цель.** Задачей настоящей работы было получение селективной системы на основе вируса M1 дрожжей *Saccharomyces cerevisiae*. **Методы.** Для создания штамма-реципиента фрагмент ДНК, кодирующий киллер-токсин вируса M1 под контролем регулируемого промотора гена *GAL1*, был встроен в геном штаммов Y-1236 и Y-2177 *S. cerevisiae*, чувствительных к токсинам. **Результаты.** Интеграция такой экспрессионной кассеты приводит к появлению условной летальности, а именно, данные штаммы гибнут на среде с галактозой, когда происходит синтез киллер-токсина. Для трансформации полученных штаммов используется линейный фрагмент ДНК, содержащий ген интереса, фланкированный последовательностями, гомологичными промотору гена *GAL1* и терминаторной области гена *CYC1*. При трансформации за счет гомологичной рекомбинации происходит выщепление последовательности, кодирующей киллер-токсин, и трансформанты растут на среде с галактозой. **Выводы.** Предложенная селективная система сочетает в себе основные преимущества других систем: возможность применения простых сред без необходимости добавления дорогостоящих антибиотиков и наличие упрощенных методик конструирования экспрессионных кассет и отбора трансформантов.

❀ **Ключевые слова:** дрожжи *Saccharomyces cerevisiae*; киллер-токсины; вирусы M1 и M28; селективные маркеры.

INTRODUCTION

Yeasts are a heterogeneous group of microorganisms, currently attracting the attention of many scientists, due to their wide biotechnological applications ranging from the food and pharmaceutical industries to the production of biofuels [1–5].

Saccharomyces cerevisiae is an ideal genetic model of yeasts, with a large number of methods in genetic engineering, molecular biology, biochemistry and protein extraction and purification developed during the process of studying this microorganism [6]. Additionally, the large number of selection markers and corresponding recipient strains allow for the insertion of specific DNA sequences into the genome [7]. In fact, *S. cerevisiae* is listed as Generally Recognized As Safe (GRAS) organism, with an ability of growth and fermentation at low pH [8]. Genetically modified yeast cells serve as biofactories producing efficacious microbial and medical products [9]. Nevertheless, one of the major drawbacks of the already engineered production strains is antibiotic resistance genes in plasmid vectors, leading to the emergence of antibiotic resistance in microorganisms [10]. Therefore, the search of new antibiotic-independent yeast selection markers is currently considered a remarkably urgent task, and in this regard, using mycotoxins of killer yeasts as a selection marker is a promising alternative approach.

Mycotoxins were first detected in *S. cerevisiae*, and they were known as killer factors [11]. These factors are either simple proteins or glycoproteins; they exhibit various structural conformations, and inhibit the growth of sensitive strains upon binding to cellular receptors at the yeast cell wall, ultimately leading to cell death. Interestingly, yeast strains are resistant to their own killer toxins, and their mycotoxins are targeted on members of the same species, as well as a wide spectrum of eukaryotic and prokaryotic organisms.

Killer toxins have been found in more than 20 genera of yeasts, especially in *Hanseniaspora, Pichia, Saccharomyces, Torulaspora, Ustilago, Williopsis* and others [13].

Key biological aspects of killer toxins have best been studied in *S. cerevisiae* such as the metabolic pathways of biosynthesis, the mechanisms of action on sensitive cells and the immunity of the killer cells. It has been reported that killer toxins are synthesized in *S. cerevisiae* in the presence of dsRNA viruses. The viruses providing the killer phenotype belong to the *Totiviridae* family and the *Mycovirus* class. One particular virus is the L-A helper virus which provides the necessary machinery for the synthesis of viral envelope, another is the satellite virus, one of the dsRNA M viruses (M1, M2 and M28, Mlus) coding for toxins K1, K2, K28 and Klus, respectively [11]. Both the helper and the satellite viruses are necessary for an effective synthesis of the killer toxin and giving rise to immunity in the host cells.

The synthesis and post-translational modifications of the killer toxins K1 and K28 has been studied and reported in the papers [14–16]. Basically, killer toxins are synthesized in the form of precursor proteins (pre-pro-peptides) in the cytoplasm, and then translocated into the endoplasmic reticulum (ER), thanks to the signal peptides (pre). In the ER, the newly-synthesized precursor proteins undergo signal cleavage, disulfide bond formation and glycosylation. In Golgi apparatus, however, the (pro) sequence is key to define the correct folding and it is ultimately cleaved, together with γ subunit.

Mature killer toxins are secreted into the extracellular medium with various mechanisms of action on sensitive cells [17]. At low concentrations, killer toxin K1 can trigger programmed cell death. Killer toxin K1 acts on the following targets: the protein receptor of the killer toxin Kre1p, potassium channel protein Tok1p and the mitochondrial protein Dnm1p, resulting in high levels of reactive oxygen species (ROS) in the targeted cells and initiating apoptosis. At high concentrations, K1 toxin forms membrane channels in target cells leading to cell necrosis [18]. Toxin K28 enters target cells through endocytosis, and moves against the secretory pathway towards the cytoplasm, where cleavage into α and β subunits occurs. The former subunit is directed towards the nucleus inhibiting DNA synthesis and cell division [19].

In this work, we have developed a selection system in order to select transformed *S. cerevisiae* yeast cells, and killer toxin DNA sequences have

been deployed as selection markers. The application of killer toxins as selection markers can greatly expand the spectrum of biotechnologically important yeast species and facilitate the process of acquiring the desired production strains without vectors carrying antibiotic-resistant genes.

MATERIALS AND METHODS

Primers

All the primers used in this work are listed in table 1.

Plasmids

Plasmids pEX-A128-M1 and pEX-A128-M28 were constructed as follows (Eurofins Genomcis, Germany). The DNA sequences of M1 and M28 viruses were flanked by BamHI/EcoRI and HindIII/EcoRI restriction sites, respectively, and cloned into the multi-cloning site of pEX-A128 plasmid. The DNA genomes of both M1 and M28 were sequenced according to Sanger method.

The construction of pAL2T-delleu2 and pAL2T-dellura3 plasmids: The two sequences flanking *LEU2* gene were amplified from the genomic DNA of *S. cerevisae* Y-1236 strain. The two primer sets ScLEU2-5'-AvrII-F/ScLEU2-5'-AflII-R and ScLEU2-3'-AflII-F/ScLEU2-3'-AvrII-R were used for this purpose. The two received DNA fragments were joined together using ScLEU2-5'-AvrII-F and ScLEU2-3'-AvrII-R primers producing one fragment, we called delleu2, containing both the 5' and 3' ends of *LEU2* gene. delleu2 fragment was then inserted in pAL2-T plasmid (Evrogen, Russia) using TA cloning technique. pAL2T-delleu2 was then processed with AflII restriction enzyme and then dephosphorylated.

We have previously designed pPICZ-FLP (Appendix 2), containing flippase gene under the control of *AOX1* gene promoter, zeocin antibiotic-resistance gene, in addition to two sequences of flippase recognistion target (FRT) sites separated by AflII restriction site. Using PGAL-SacI-F and PGAL-SalI-R primers and pYES2 plasmid (ThermoFisher Scientific, USA), *GAL1* gene promoter was amplified. The fragment was then processed using SacI and SalI restriction enzymes, and then inserted in pPICZ-FLP plasmid instead of *AOX1* gene promoter. The resulting plasmid pPICZ-P_{GAL1}-FLP was cut using AflII restriction enzyme and ligated into pAL2-T-delleu2 plasmid. The map of pAL2-

Table 1

The sequences of primers used in this work

Primer	5′ → 3′ sequence
ScLEU2-5′-AvrII-F	CCTAGGAGTTCGAATCTCTTAGCAACC
ScLEU2-5′-AflII-R	TCTTAAGACACCTGTAGCATCGATAGC
ScLEU2-3′-AvrII-R	CCTAGGCCAGATCATCGTTATCCAG
ScLEU2-3′-AflII-F	GTGTCTTAAGAAGTTAAGAAAATCCTTGC
ScURA3-5′-AvrII-F	CCTAGGACATGAACAAACACCAGAGTC
ScURA3-5′-AflII-R	CCTTAAGAATCAGTCAAGATATCCACATG
ScURA3-3′-AvrII-R	CCTAGGTGGATTTGGTTAGATTAGATATGG
ScURA3-3′-AflII-F	GATTCTTAAGGGATGCTAAGGTAGAGG
PGAL-SacI-F	AAATGAGCTCGATCCACTAGTACGGATTAGAAG
PGAL-SalI-R	AAATGTCGACTTAATATTCCCTATAGTGAGTCG
αTOX-F	AAATAAAGCTTATGGAAGCGCCGTGGTATGACAAGATCTG
αTOX-R	AAATTGAATTCTTAAGCAACGGTAGCGCCATTAGGATCTG
LEU2-dR	ACCTTTGGATCCTCCTTTTTCTCCTTCTT
LEU2-dF	GAGGATCCAAAGGAATACAGGTAAGCAAAT
expαTOX-BHI-F	AATAGGATCCGGCGTAACCACCACACC
expαTOX-BHI-R	AATAGGATCCCGCAAATTAAAGCCTTCG
GFP-F	GGACTACTAGCAGCTGTAATACGACTCACTATAGGGAATATGGTGAGCAAGGGC
GFP-R	TCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTACTTGTACAGCTCGTCC

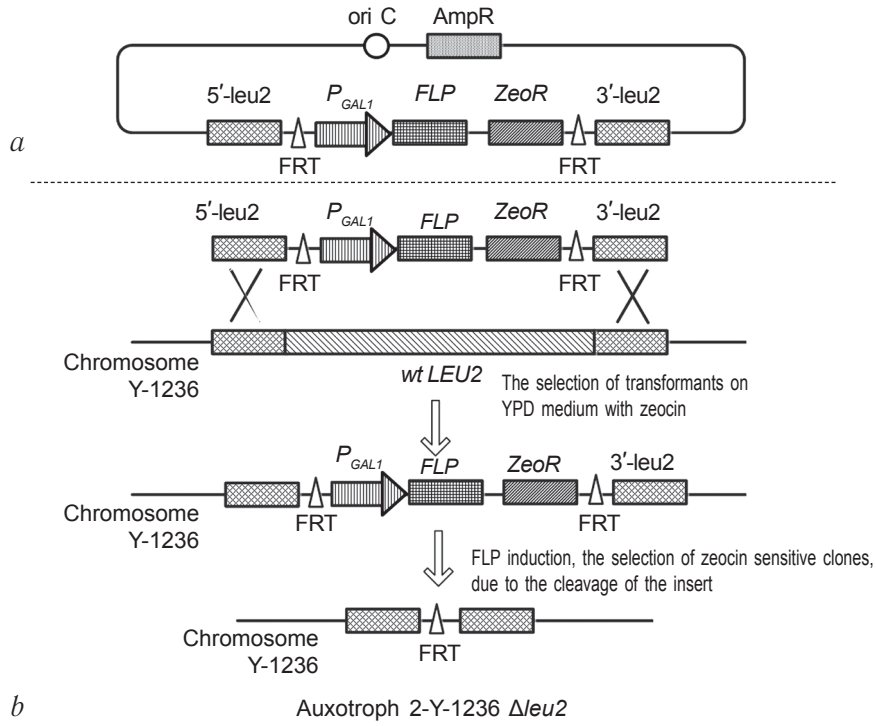

Fig. 1. Scheme for obtaining *S. cerevisiae* strains with deletions in *LEU2* и *URA3* genes: *a* – structure of plasmid pAL2T-delleu2; *b* – obtaining the auxotrophic strain 1-Y-1236 (Δ*leu2*) using the FLP-FRT recombination system (strains 2-Y-1236 (Δ*ura3*), 1-Y-2177 (Δ*leu2*) and 2-Y-2177 (Δ*ura3*) were obtained similarly). Auxotrophic strains to leucine and uracil 3-Y-1236 (Δ*leu2* Δ*ura3*) and 3-Y-2177 (Δ*leu2* Δ*ura3*) were obtained using strains with a single auxotrophy

T-delleu2 is illustrated in figure 1 *a*. In the same manner, we constructed pAL-2-T-delura plasmid, using the two sets of primers ScURA3-5'-AvrII-F/ScURA3-5'-AflII-R and ScURA3-3'-AflII-F/ScURA3-3'-AvrII-R. We have checked the structure of the constructed plasmids using PCR and restriction analysis.

The construction of pYES2-M1 and pYES2-M28 plasmids: Plasmids pEX-A128-M1 and pEX-A128-M28 contained the DNA fragments of M1 and M28 viruses genomes flanked by the two restriction sites BamHI/EcoRI and HindIII/EcoRI, respectively. These DNA fragments were cut and inserted into pYES2 using their corresponding restriction sites. In both pYES2-M1 and pYES2-M28 plasmids the whole DNA sequences of M1 and M28 viruses were located under the control of *GAL1* gene promoter, the expression of which is induced by galactose in the growth medium.

The construction of pAL2-T-P_{GAL1}-aTOX plasmids: *LEU2* gene was first amplified using the primers ScLEU2-5'-AvrII-F/LEU2-dR and LEU2-dF/ScLEU2-3'-AvrII-R and *S. cerevisiae* Y-1236 chromosomal DNA as the DNA matrix. Then, *LEU2* DNA fragments were purified and PCR-amplified using ScLEU2-5'-AvrII-F and ScLEU2-3'-AvrII-R primers. The resulting fragment was inserted into pAL2-T (Evrogen, Russia) using TA-cloning technique. As a result, pAL2-T-LEU2 was constructed containing the full sequence of *LEU2* gene, with BamHI restriction site at its 3' end. Next, we used pEX-A128-M1 plasmid as the DNA matrix for amplifying M1 toxin gene using aTOX-F and aTOX-R primers. The amplified fragment was then processed with HindIII and EcoRI restriction enzymes and inserted into pYES2 vector. The resulting plasmid pYES2-aTOX was used as the DNA matrix for PCR-amplification using expaTOX-BHI-F and expaTOX-BHI-R primers. The amplified fragment was processed with BamHI restriction enzyme and inserted into pAL2-T-LEU2 plasmid. The resulting plasmid pAL2-T- P_{GAL1}-aTOX contained the DNA sequence of M1 virus toxin under the control of *GAL1* promoter, with *LEU2* gene located at the 3' end (figure 2, *a*).

Strains

S. cerevisiae yeast strains used in this work are listed in table 1, in addition to the bacterial strain *Escherichia coli* DH5α (*fhuA2* Δ(*argF-lacZ*)

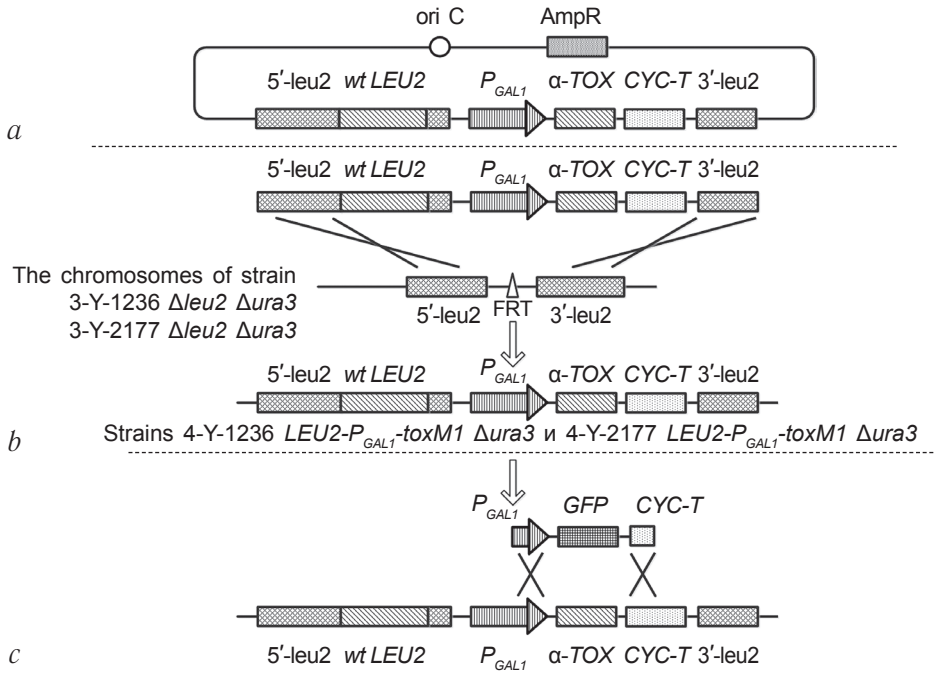

Fig. 2. Scheme of M1 toxin fragment integration into the genomes of *S. cerevisiae* strains and its use as a selection marker: *a* – structure of plasmid pAL2-T-P_{GAL1}-αTOX; *b* – obtaining the 4-Y-1236 (*LEU2-*P_{GAL1}*-toxM1 Δura3*) and 4-Y-2177 (*LEU2-*P_{GAL1}*-toxM1 Δura3*) strains in the genome of which the toxin sequence of the M1 virus was integrated under the control of the regulated promoter of *GAL1* gene; *c* – integration of the *GFP* gene flanked by sequences homologous to the promoter of the *GAL1* gene and the termination region of the *CYC1* gene

U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17.

Media and growth conditions

For the cultivation of the yeast strains we used the following media: **YPD**: 2% glucose, 2% peptone, 1% yeast extract, 2.4% agar. **YPDS**: 2% glucose, 2% peptone, 1% yeast extract, 2.4% agar, 1M sorbitol, 200mg/ml zeocin. **MD** and **MGal** (minimal media): 7.34 mM KH_2PO_4, 0.95 mM $K_2HPO_4 \cdot 2H_2O$, 4 mM $MgSO_4 \cdot 7H_2O$, 0.9 mM $CaCl_2$, 1.7 mM NaCl, 37.85 mM $(NH_4)_2SO_4$; vitamins and microelements; 2% – glucose (MD) or galactose (MGal), 2.4% agar, amino acids and nitrogen base (if necessary), lycine, uracil – 40 mg/l. For the cultivation of bacteria, we used LB medium: 1% tryptone, 0.5% eyast extract, 170 mM NaCl, $5 \cdot 10^5$ benzylpenicillin μg/mL. *S. cerevisiae* strains were grown at 30 °C and *E. coli* at 37 °C.

Table 2

***S. cerevisiae* yeast strains used in this work**

Strain	Genotype	Source
Y-1236	*MATa wt*	Genetika
1-Y-1236	*MATa Δleu2*	This work
2-Y-1236	*MATa Δura3*	This work
3-Y-1236	*MATa Δleu2 Δura3*	This work
Y-2177	*MATa wt*	Genetika
1-Y-2177	*MATa Δleu2*	This work
2-Y-2177	*MATa Δura3*	This work
3-Y-2177	*MATa Δleu2 Δura3*	This work
4-Y-1236	*MATa LEU2-*P_{GAL1}*-toxM1 Δura3*	This work
4-Y-2177	*MATa LEU2-*P_{GAL1}*-toxM1 Δura3*	This work

Molecular biology methods

In this work we used FastAP alkaline phosphatase (ThermoFisher Scientific, USA) and T4 ligase (Evrogen, Russia) according to the manufacturer's instructions. DNA purification from agarose gel and reaction mixtures was performed using Cleanup Standard kit (Evrogen, Russia). For plasmid extraction we used Plasmid Miniprep kit (Evrogen, Russia). PCR was conducted using Encyclo Plus PCR kit (Evrogen, Russia). Gel electrophoresis of the DNA fragments was performed in 0.7% agarose in TAE buffer [20]. Transformation procedures in *E. coli* and *S. cerevisiae* were performed according to [21, 22]. DNA extraction from *S. cerevisiae* was performed according to [23]. DNA sequencing was carried out utilizing BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA).

RESULTS

Engineering 3-Y-1236 and 3-Y-2177 strains with *LEU2* and *URA3* gene deletions

Both prototrophic strains *S.cerevisiae* Y-2177 and Y-1236 supersensitive to killer toxins were obtained from the Russian Federal Institution "State Research Institute of Genetics and Selection of Industrial Microorganisms of the National Research Center" of Kurchatov Institute (Genetika) (http://eng.genetika.ru). At first, we performed *URA3* и *LEU2* gene deletions in both *S.cerevisiae* strains Y-2177 and Y-1236, respectively. For this purpose, we used the two plasmids pAL2T-delura3 and pAL2T-delleu2.

The DNA fragment 5'LEU2-FRT-P_{GAL1}-FLP-ZeoR-FRT-3'LEU2 was amplified using the primers ScLEU2-5'-AvrII-F and ScLEU2-3'-AvrII-R, with pAL2T-delleu2 plasmid as the matrix DNA. The DNA fragment was purified and transformed into both Y-2177 and Y-1236 strains, and the transformants were selected on YPDS agar plates with the antibiotic zeocin. Due to homologous recombination during the process of transformation, *LEU2* coding sequence was replaced by FRT-P_{GAL1}-FLP-ZeoR-FRT cassette. Next, we incubated the transformed clones in galactose-containing MGal liquid media for 24 hours. Flippase was being synthesized in these cells causing double strand breaks in FRT sequence, resulting in the deletion of FRT-P_{GAL1}-FLP-ZeoR-FRT cassette from the transformants genomes. Subsequently, only the cells with cassette deletion showed growth on YPDS agar plates with zeocin. At this stage, we used the sterile velveteen press method to perform replica plating, where the transformants were transferred onto fresh YPD agar plates. The resulting strains 1-Y-1236 (Δ*leu2*) and 1-Y-2177 (Δ*leu2)* did not grow on MD medium lacking leucine, indicating auxotrophy. The scheme of the experiments is shown in figure 1, *b*.

In the same manner we obtained both 2-Y-1236 (Δ*ura3*) and 2-Y-2177 (Δ*ura3*) uracil auxotrophic strains based on pAL2T-delura3 plasmid. Later we engineered the strains 3-Y-1236 (Δ*leu2* Δ*ura3*) and 3-Y-2177 (Δ*leu2* Δ*ura3*) auxotrophic to both leucine and uracil. The obtained deletions were confirmed using PCR with the chromosomal DNA of both 3-Y-1236 and 3-Y-2177 strains used as the matrix DNA and ScLEU2-5'-AvrII-F/ScLEU2-3'-AvrII-R primers, while Y-1236 and Y-2177 strains served as a positive control, with ScURA3-5'-AvrII-F/ScURA3-3'-AvrII-R primers.

Expressing the full genome of M1 and M28 viruses in 3-Y-1236 and 3-Y-2177 strains

pYES2-M1 and pYES2-M2 vectors were used to transform 3-Y-1236 (Δ*leu2* Δ*ura3*) and 3-Y-2177 (Δ*leu2* Δ*ura3)* strains. The transformants were chosen according to uracil prototrophy, then we analyzed the emergence of killer toxin effect. For this purpose, the following strains were plated on MGal agar plates: 3-Y-1236 (Δ*leu2* pYES2-M1), 3-Y-1236 (Δ*leu2* pYES2-M28), 3-Y-2177 (Δ*leu2* pYES2-M1) and 3-Y-2177 (Δ*leu2* pYES2-M28). The medium contained galactose as a sole carbon source, and as a negative control we plated 3-Y-1236 (Δ*leu2* pYES2) and 3-Y-2177 (Δ*leu2* pYES2) strains, each containing pYES2 plasmid.

On the media containing galactose, killer toxin proteins were being synthesized, inhibiting the growth of sensitive strains and lysis zones were consequently formed. Results are shown in figure 3. The expression of the full viral genome did not affect the growth of the strains: 3-Y-1236 (Δ*leu2* pYES2-M1), 3-Y-1236 (Δ*leu2* pYES2-M28), 3-Y-2177 (Δ*leu2* pYES2-M1) and 3-Y-2177

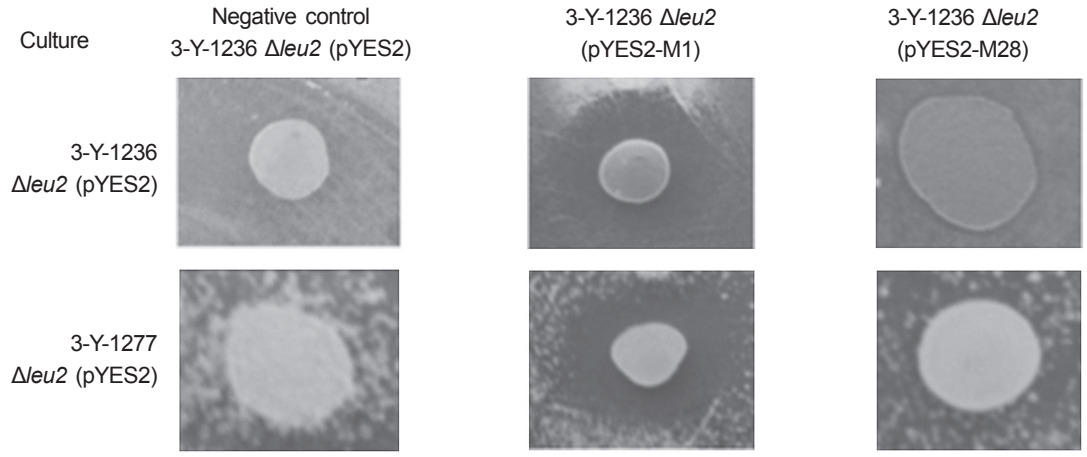

Fig. 3. Phenotypes of strains 3-Y-1236 (Δ*leu2* pYES2-M1) and 3-Y-1236 (Δ*leu2* pYES2-M28) on a medium with lawns of the control strains 3-Y-1236 (Δ*leu2* pYES2) and 3-Y-2177 (Δ*leu2* pYES2). The inhibition zone is the result of the killer toxins action

(Δ*leu2* pYES2-M28), since these strains are resistant against M1 and M28 toxins, respectively [11].

The most pronounced killer effect was manifested in strain 3-Y-1236 (Δleu2 pYES2-M1) synthesizing the M1 virus. Therefore, M1 toxin was precisely chosen for the further development of the selective system.

Using the M1 virus sequence as a selection marker

The DNA fragment of the M1 killer toxin (a-TOX 315 bp) previously used by (Gier, et al) was amplified using the primer pair a-TOX-F and a-TOX-R [24]. Consequently, we constructed the plasmid pAL2-T-P_{GAL1}-aTOX carrying one fragment of M1 virus (a-TOX fragment) under the control of *GAL1* gene promoter. This plasmid was used as a matrix, and the primer pair ScLEU2-5'-AvrII-F and ScLEU2-3'-AvrII-R was used to amplify the fragment 5'LEU2-P_{GAL1}-aTOX-3'LEU2 3'. The resulting fragment was transformed into 3-Y-1236 and 3-Y-2177 strains, and the transformants were chosen according to their leucine prototrophy recovery. The scheme of the experiment is illustrated in figure 2, *b*. As a result, the two strains 4-Y-1236 (*LEU2-*P_{GAL1}*-toxM1 Δura3*) and 4-Y-2177 (*LEU2-*P_{GAL1}*-toxM1 Δura3*) were obtained. These two strains are characterized by conditional lethality, i.e. the strains did not grow on media containing galactose, as long as the toxin was synthesized in the cells (figure 4). The integration of the fragment into the genome was checked using PCR, with the genomes of 4-Y-1236 and 4-Y-2177 transformants as the matrix DNA and aTOX-F/aTOX-R primer pair. In case of 4-Y-1236 strain showing an insignificant inhibition of growth on the glucose-containing medium, this may be due to the peculiarities of the regulation of glucose repression in this strain. For our further work we used 4-Y-2177 strain.

Since 4-Y-2177 growth is inhibited on media with galactose, as a result of toxin synthesis, therefore the toxin gene can be used as a selection marker. In order to evaluate the feasibility of this approach in selecting transformants, we used GFP.

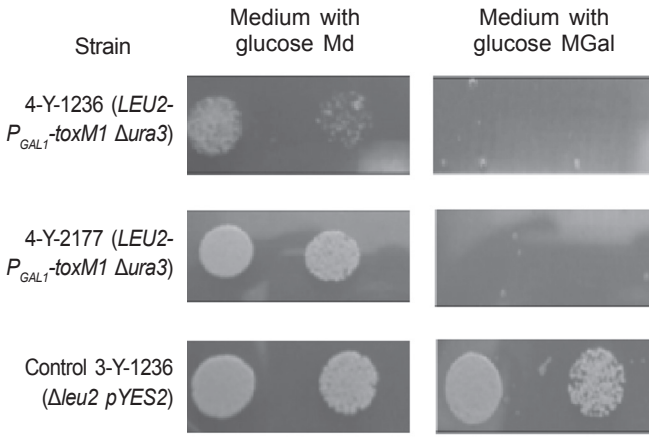

Fig. 4. Growth of 4-Y-1236 (*LEU2-*P_{GAL1}*-toxM1 Δura3*) and 4-Y-2177 (*LEU2-*P_{GAL1}*-toxM1 Δura3*) strains in the genome of which, the M1 virus toxin sequence was integrated under the control of the regulated promoter of the *GAL1* gene on media with glucose and galactose. The strains are characterized by conditional lethality – they do not grow on media with galactose, since toxin synthesis occurs in their cells under these conditions. 10 µl of a suspension of 10^4 and 10^3 cells/ml was plated on each agar medium

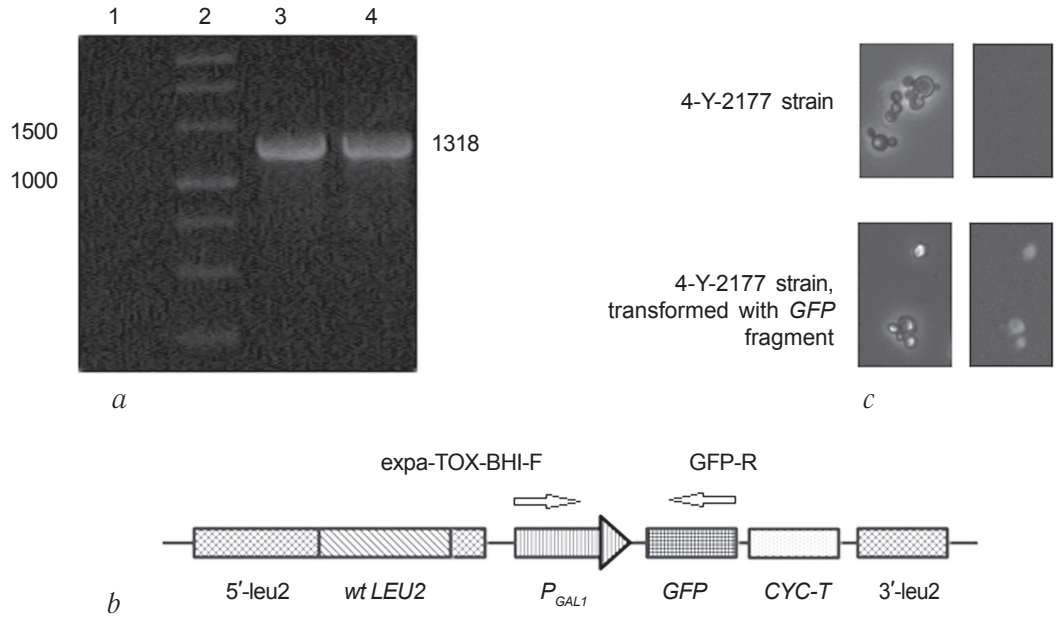

Fig. 5. Evaluation of *GFP* gene integration in transformants genome: *a* – results of PCR with expaTOX-BHI-F and GFP-R primers and genomic DNA of 1) 4-Y-2177 strain; 3) 4-Y-2177 strain transformed with *GFP* fragment, clone 1; 4) 4-Y-2177 strain transformed with *GFP* fragment of clone 2. The sizes of the fragments correspond to the theoretically expected 1343 bp. 2) Evrogen DNA length marker 1 kb; *b* – scheme representing the location of primers; *c* – fluorescence microscopy results of the transformed cells in comparison to untransformed 4-Y-2177 strain

We amplified GFP gene using GFP-F and GFP-R primers, which at their 5'contained homologous sequences to *GAL1* gene promoter and *CYC1* gene terminator region. The resulting amplified DNA fragment was purified and transformed into 4-Y-2177 strain, and MGal medium was used to select the transformants. During transformation, and due to homologous recombination, the sequence coding for the killer toxin was replaced by GFP gene sequence (figure 2, *c*). The resulting transformants were able to grow on the galactose-containing medium MGal, giving the evidence of replacing the toxin gene sequence by the GFP gene sequence. In order to confirm the integration of GFP gene into the genomes of the transformants, PCR was performed using expaTOX-BHI-F and GFP-R primer pair, and fluorescent microscopy was (figure 5).

DISCUSSION

S. cerevisiae yeasts are widely used in various biotechnological processes. In fact, various transformation methods with practical transformant selection systems largely contribute to the creation of new industrially-relevant production yeast strains.

As for *S. cerevisiae,* genes such as *URA3, LEU2, HIS3, TRP1* and others, coding for various enzymes in yeast metabolic pathways are traditionally used for plasmid construction. Naturally, mutations in these genes lead to auxotrophy of the corresponding amino acid [25]. As a general rule, plasmids used to genetically engineer production strains carry bacterial antibiotic resistance genes. This feature is especially practical for amplifying the plasmids in *E. coli* [26]. Furthermore, antibiotic resistance genes such as zeocin, are used for the direct selection of yeast transformants [27].

However, during the process of engineering a production strain, the presence of antibiotic resistance genes in plasmids is considered a major problem. The use of antibiotics for maintaining the plasmids is not recommended for the synthesis of recombinant proteins in the pharmaceutical industry. The reason for this disapproval is the potential risk of contaminating the finished pharmaceutical products with antibiotics, and contaminating the environment with the DNA sequences coding for antibiotic resistance genes be means of horizontal gene transfer [28, 29].

In this regard, in bacteria, for example, attempts are being made to replace the antibiotic

resistance genes in plasmids with the lgt gene encoding (pro) lipoprotein glyceryl transferase. This DNA fragment is involved in the biosynthesis of bacterial lipoprotease, and the deletion of this gene is lethal. One system has been developed for obtaining production strains of recombinant proteins, in which lgt gene has been deleted from the bacterial chromosome and inserted in a plasmid, together with a target gene. The loss of the plasmid resulted in cell death [30].

For the first time, in this work we used the yeast killer toxin M1 as a selection marker, allowing its application in engineering production strains, in the absence of DNA gene sequences coding for antibiotic resistance mechanisms.

This system of selection is designed to function in recipient strains, which are sensitive to the killer toxin.

Firstly, in case of strain prototrophy, *URA3* and *LEU2* genes deletions are introduced into their genomes. Then, a construct containing the DNA sequence of M1 virus toxin is integrated under the control of *GAL1* gene promoter. In order to obtain a production strain, it is necessary to transform the recipient strain with the amplified coding sequence of the gene of interest, flanked by *GAL1* gene promoter and terminator. Because of homologous recombination, the gene encoding the toxin is replaced and target transformants are selected on galactose-containing media. Accordingly, the gene of interest becomes a part of the transformed strains genomes, under the control of *GAL1* gene promoter, providing a regulated expression pattern. This approach can be used for the expression of recombinant proteins, helping reduce the spread of antibiotic resistance genes, the release of antibiotics into the environment and freeing finished products (usually used in pharmaceuticals) from potentially harmful antimicrobial residues. Besides, this selection system can be used as an approach to solve the problem of the shortage of handy selection markers emerging from working with new yeast strains.

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Appendix 1

DNA sequence of M1:

GAAAAATAAAGAA**ATG**ACGAAGCCAACCCAAGTATTAGTTAGATCCGTCAGTATATTATTTTTCATCACATTACTAC
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GGGAGACGAATTACTATAAATTGTATTGGAGTACTGGCCAGTGGATAATGTCGATGAAGTTTATTGAGGAAAGTATT
GATAACGCCAATAATGACTTTGAAGGCTGTGACACAGGCCAC**TAG**GGCATCGTGTCTGACCTCTGATGCGATAACT
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DNA sequence of M28:

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CAACAGGCAATTACTACAGAGACGTTAACCTCTGTGGTGACGACGAGGCAAGGTGCCACGATGAGCTACGC**TAA**
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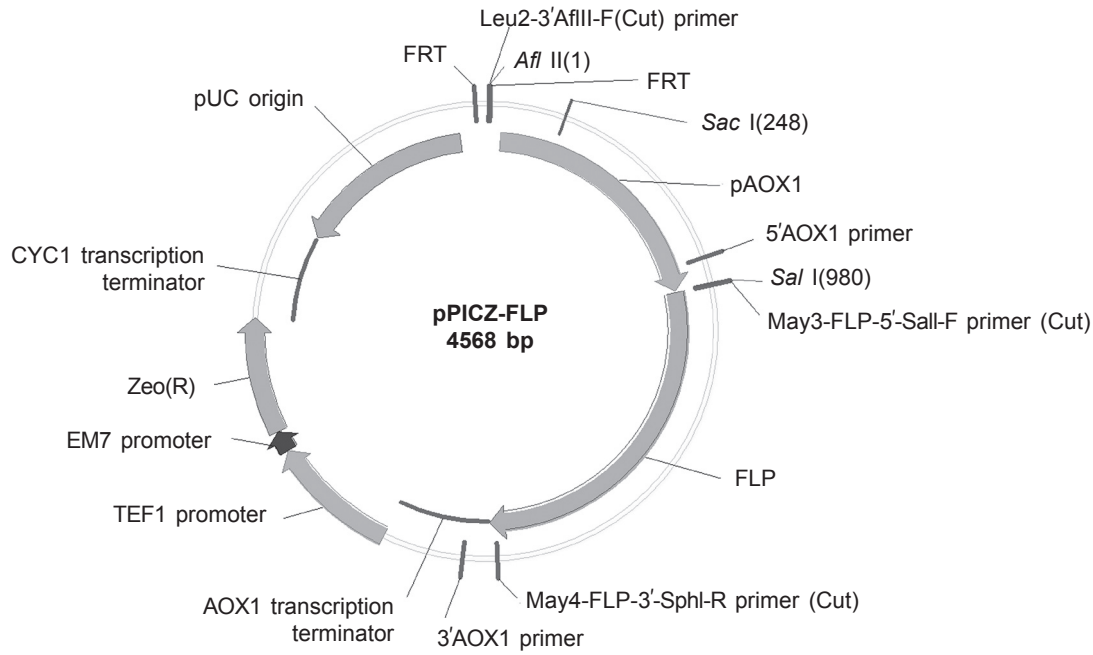
Appendix 2

pPICZ-FLP plasmid map:



Nucleotide sequence of pPICZ-FLP plasmid:

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