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EXPERIMENTAL ARTICLES

Application of the New *ccdAB*-Type Natural Toxin–Antitoxin Module for Stabilization of Inheritance of Expressive Plasmid Vectors Based on the Bacteriophage N15 Replicon in *Escherichia coli* Cells

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Abstract—Biopharmaceutical industry currently produces considerable quantity of novel recombinant preparations by way of overexpression in *Escherichia coli* cells, an inexpensive, efficient, time-proven, and practically feasible system of heterologous expression. Due to the instability of maintenance and inheritance of expression vectors in producer cells, the cells that have spontaneously lost the plasmid gain a significant selective advantage over the cells producing a heterologous protein and accumulate in the fermentor. For solution of this problem, it is proposed to develop a new generation of expression vectors with high stability of inheritance in the absence of external selective pressure, using a replicon of phage N15, which possesses its own system for active distribution of plasmid copies in the daughter cells, supplemented by a toxin–antitoxin genetic module preventing the loss of a plasmid. Two new addiction modules homologous to the known *ccdAB* and *mazEF* systems were isolated from natural enterobacterial populations and characterized. The testing showed more effective operation of the *ccdAB* module. The latter was a basis for construction of new expression vectors pN15E41 and pN15E61 demonstrating the high synergism of action of the plasmid segregation systems and the addiction module and directly applicable for biotechnological practice.

Key words: plasmid inheritance, phage N15 replicon, toxin–antitoxin modules, addiction systems, expression vectors

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One of the important achievements of modern biotechnology is creation and rapid development in recent decades of a novel area in the world economy: the biopharmaceutical industry.

Various interferons, erythropoietins, growth factors, antibodies, vaccine proteins, and commercial enzymes are produced in the volumes of up to several tons per year and have become a substantial part of everyday practice in modern medicine, food and pharmaceutical industries. Many of these proteins can be obtained by means of overexpression in (*Escherichia coli*) cells, which are an inexpensive, efficient, time-proved and practically feasible system of heterologous expression.

Because of probable instability of maintenance and inheritance of the target expression vectors in producer cells, the cells that have spontaneously lost the plasmid (expression vector) gain a considerable selective advantage over the cells preserving the ability to produce the required heterologous protein. Accumulation in commercial cultures of large quantities of inactive "empty" cells reducing the product yield is most often prevented by supporting selection with antibiotics introduced into the nutrient medium in the fermentor.

A problem therefore arises of purification of the end product from antibiotics, since they may promote the emergence and dissemination of antibiotic-resistant microorganisms, including pathogenic ones, and have a negative effect on the human organism, in particular, inducing sensitization resulting in allergic reactions [1, 2]. For solution of this problem, it is proposed to create a new generation of expression vectors with high stability of inheritance in the absence of external selective pressure.

Most of the currently used plasmid expression vectors carry replicons randomly distributed among the daughter cells after division. As a result of such statistical distribution, there is a certain probability of formation of plasmid-free descendants [3].

In the present project, it is proposed to overcome this potential difficulty by using the replicons that possess their own systems of active distribution of plasmid DNA copies in daughter cells (plasmid segregation)

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for designing new vectors [4, 5]. These replicons are different natural low-copy plasmids including the prophage of the moderate bacteriophage N15. The uniqueness of this object is that the N15 prophage in the lysogenic state is not integrated into the bacterial host chromosome but is a linear low-copy plasmid with covalently closed "pin" telomeres, the only known linear plasmid of *E. coli* [6].

However, even with the vectors based on these replicons, plasmid-free cells may appear, though far less frequently, e.g., as a result of spontaneous mutations in the region of origin of replication or in the genes of proteins of the segregating mechanism. For enhancing the vector maintenance in the cells of producer bacteria, it is advisable to insert additional genetic modules preventing their loss in the new N15 replicon-based expression vectors.

One of the types of such modules occurring in naturally originating bacterial plasmids are the so-called addiction systems, sometimes also termed toxin–antitoxin modules [7, 8]. These genetic elements are an operon carrying genes encoding a protein toxin and an antitoxin, which may be either of protein or nonprotein nature. Antitoxin is always produced in a quantity exceeding the quantity of toxin. In cells, the toxin is more stable than the antitoxin; therefore, in the case of spontaneous loss of a plasmid bearing this genetic module, the toxin that has lost its inhibitor interacts with its cell target, killing the cell. At least 6 different types of toxin–antitoxin modules are known for *E. coli*. They have been described in some moderate phages, which are present as a plasmid for some part of their life cycle in the cells (e.g., the *E. coli* phage P1).

At the moment, the following most studied genetic modules are known that cause postsegregational death of *E. coli* cells that have lost the module-carrying genetic element during division: *ccdAB* localized at the F sex plasmid (the toxic gene product inhibits DNA gyrase), *phd-doc* localized at prophage P1 present in the cell as a plasmid (localization of the toxic effect of the toxin gene product has not been determined: cell death occurs as a result of inhibition of protein biosynthesis), and *mazEF* localized in the bacterial genome (localization of the effect of toxin gene has not been determined). Recent works of Israeli researchers [9] have shown synergetic effect between the *phd-doc* and *mazEF* systems. We have decided to focus on the search of the above modules and their isolation from natural objects, as they are candidates for the best stabilizing effect in the *E. coli*–expression vector system.

In spite of the fact that the modules stabilizing *E. coli* plasmids are rather widespread in nature, the search for new variants of them with a more active stabilizing effect on the genetically engineered *E. coli* strains used in biotechnology requires analysis of an ample quantity of sources with a maximally great biological diversity of *E. coli* and other coliform bacterial strains under high life density of a bacterial community. Since *E. coli* and coliform bacteria are normal saprophytic symbionts of the intestines of mammals, horse feces and feces-polluted wastewater, are, in our opinion, the most promising objects of this kind. The fact that horses are not ruminants and bacterial biomass from some parts of its stomach is not digested increases considerably the total titer of coliform bacteria in the feces. Wastewater also contains an ample quantity of coliform bacteria potentially carrying addiction modules, but life density along with the general level of biodiversity is much lower in wastewaters than in horse feces. According to the literature data, about 30% of natural coliforms maintain some or other plasmids. The foreign authors who have originally described addition modules place special emphasis on the difficulties of searching for these elements in natural objects. These authors, however, did not investigate the bacterial communities of feces.

Some data [10] give evidence of association between the natural determinants of resistance of enterobacteria to fluoroquinolones, which per se have chromosomal localization, and the presence of plasmids carrying the addiction systems. Obviously, this is due to the fact that plasmid-selected resistance to the protein toxin blocking DNA gyrase may provide cross resistance to antibiotics affecting the same target. This circumstance may considerably facilitate the search of the new variants of addiction modules.

Thus, it is optimal to apply several different stabilizing systems in one vector simultaneously to achieve reliable plasmid maintenance under the conditions of biotechnological production without selection with antibiotics.

The goal of this work was to search in the natural populations of enterobacteria for the new variants of toxin–antitoxin modules that could be used for stabilization of commercial expression vector systems and that would provide better inheritance of the vector compared to the previously known modules.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. Strains *E. coli* C (Sasaki and Bertani, 1965) and MC1061 [11] were used as a host for phage reproduction; DH10B [12] was used in all cloning experiments.

Bacterial cultures were grown under aeration at 37°C in LB medium with chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), ampicillin (100 μg/ml), or tetracycline (20 μg/ml). *Pfu* polymerase (Promega), T4 DNA ligase (NBI Fermentas), T4 polynucleotide kinase (Sibenzyme), and restriction endonucleases (Promega, New England Biolabs, Sibenzyme) were used in accordance with the manufacturers' recommendations. All PCR amplifications were carried out with *Pfu* DNA polymerase (Promega). *Taq* polymerase (Sigma) was used for the screening of plasmid DNA preparations for the presence of toxin–antitoxin cassettes of the known types. The following synthetic oligonucleotides were used:

FlccdABD: 5'-TGACGCGATCACCCGACT-3'
FlccdABR: 5'-CAGTCCTGGATATGCTCAAT-3'
ccdABD: 5'-ATGAAGCAGCGTATTACTGTC-3'
ccdABR: 5'-TTATATTCCCCAGAACATCAG-3'
phd-docpD: 5'-TGGTGCTTTATGCCTGTGA-3'
phd-docR: 5'-CTACTCCGCAGAACCATACAAT-3'
phd-docD: 5'-GTGTTTATGCAATCCATTAACTTC-3'
mazEFD: 5'-CTACCCAATCAGTACGTTAATT
mazEFR: 5'-GGTTTGAGGAAAGGGTTAT
Km-Pac: 5'-GTTAATTAACGTTGTGTCTCAAAATC
Km-Not: 5'-CGCGGCCGCCGTCCCGTCAAGT

Procedure for the isolation of enterobacteria. Animal feces samples were resuspended in the buffer (pH 7.2) containing *tris*-HCl, 50 mM and NaCl, 0.09% at 1 : 4 (wt/vol) ratio and incubated on an orbital shaker at room temperature during 20 min for desorption of bacteria from solid particles. Then the suspensions were allowed to stand for 5 min for precipitation of rough solid particles and a 50-μl aliquot was taken from the supernatant. Dilutions for inoculation were prepared. In most cases, inoculation of 50 μl from the 10^2 dilution yielded 100–200 colonies per plate. If necessary, different dilutions were prepared and plated. Wastewater samples were plated without dilution or diluted with the same buffer to obtain the optimal quantity of colonies on the plates.

Inoculation for the primary isolation of coliform enterobacteria was made as described in [13] onto the plates with LTA (lauryl-tryptose agar) medium containing the following: tryptose (Difco), 20.0 g; lactose, 5.0 g; NaCl, 5.0 g; K_2HPO_4, 75 g; KH_2PO_4, 2.75 g; sodium dodecyl sulfate, 0.1 g; agar, 15 g; and water, up to 1 l; pH 6.8 ± 0.2. This medium is selective for coliform bacteria. The plates were incubated at 37°C overnight. The plates with 10–200 and 300–1000 colonies were used for obtaining individual isolates of coliform bacteria and preparing washouts, respectively.

DNA isolation, electrophoresis, and Southern blot analysis. Purification of DNA fragments from agarose gel and isolation of the plasmid DNA were carried out using commercial Qiagen kits. Total DNA from *E. coli* cells was isolated according to the scheme proposed in [6]. The standard methods of electrophoresis in agarose gel and plasmid DNA transformation were used.

Plasmid DNA was obtained from the natural isolates of enterobacteria using phenol extraction [14] to prevent the loss of DNA of large plasmids.

Identification of the complete nucleotide sequence of plasmid-stabilizing modules. The complete sequences of genetic modules contained in the inserts of selected clones were identified by the Sanger method of sequencing with an AbiPrism automatic sequencer (Applied Biosystems, United States), using standard kits as recommended by the manufacturer. The sequencing was carried out with the standard primers M13 Forward and M13 Reverse (Fermentas, Lithuania).

Analysis of the stability of plasmid inheritance. The efficiency of stabilization of plasmid inheritance by the selected toxin–antitoxin modules was tested using a two-plasmid system, the function of which was based on exclusion of plasmids belonging to the same incompatibility group when they were present in the same cell under nonselective conditions. For the analysis, *E. coli* cells were transformed by the obtained plasmid DNA with simultaneous addition of the saturating concentration of the plasmid pUK21. The transformants were grown on the plates with two antibiotics: kanamycin and ampicillin. The colonies of double transformants were washed with the LB medium, diluted 100-fold, and grown in a liquid medium with the same antibiotics for 5 h for complete removal of the cells not containing the marker of ampicillin resistance. The culture obtained (1 : 100 vol/vol) was used for inoculation of the flasks with LB medium containing no antibiotics for competitive displacement of the plasmids. After three passages without antibiotics, the culture was diluted and inoculated to obtain isolated colonies on four types of plates: without antibiotics, with both antibiotics, with ampicillin, and with kanamycin. If the number of colonies on the plates with ampicillin and without antibiotics was approximately equal and exceeded more than threefold the number of colonies on the plates with kanamycin and with both antibiotics, the colonies grown on the medium with ampicillin were washed off followed by three additional transfers. Then, inoculation was repeated by the analogous procedure and the results were registered.

RESULTS AND DISCUSSION

For the quickest detection of the sought DNA sequences in the natural populations of coliform bacteria and their phages, we chose the route of screening and making the clone libraries of addiction modules after their amplification in the polymerase chain reaction directly with total DNA of the community as a template. For this purpose, we performed an extensive comparative bioinformation analysis of the multiple alignments of the nucleotide sequences of genetic modules of interest taken from the GenBank international database. The analysis revealed conservative regions of the consensus of sequences, and later these data were used to design the following universal oligonucleotide primers flanking the functional elements of the sought addiction modules.

The *ccdAB* (control cell death) module was the first of the discovered genetic elements causing the death of the segregant cells. The function of the components of this module has a considerable effect on maintenance in *Escherichia coli* cells of a large and extremely low-copy (usually not more than one copy per cell) sex plasmid F carrying the genes important for the cell (e.g., the genes of the proteins of the conjugative pili). The protein product of the *ccdA* gene (an unstable

antidote) is bound in the cell directly to the protein product of the *ccdB* gene (a toxin resistant to degradation). The full-size F plasmid contains two more modules that can cause postsegregational apoptosis of the segregants, with antisense RNAs serving as antidotes. In the case of F mini-plasmids not containing these additional modules, more than 90% of the segregants (as evidenced by the literature data) are subject to apoptosis as a result of inhibition of cellular processes by the CcdB protein. Apart from the direct inhibitory effect of CcdB on the cellular DNA gyrase, it can also induce the SOS response of a cell in the presence of active recombination systems RecA and RecBC. The studies of the *ccd* operon demonstrated that it was negatively autoregulated at the transcriptional level directly by the complex of its own protein products bound to the operator of the promoter region of the *ccdAB* module. The cell target of the toxic effect of CcdB is the alpha subunit of the tetrameric enzyme DNA gyrase in a complex with DNA with the participation of the cell chaperone GroES.

The *phd-doc* (prevents host death—death on cure) module initially belongs to coliphage P1, which is able to lysogenize *E. coli* cells as a low-copy plasmid; the frequency of prophage–plasmid losses is only 10^{-5} per cell per generation. Thus, this module is a quite efficient agent for maintenance of *E. coli* plasmids. This module is molecularly arranged as a compact operon containing both the *phd* antitoxin gene and the *doc* toxin gene. Similarly to the *ccdB* module, the protein product of the antitoxin gene is unstable, being a substrate for cellular proteases ClpPX and Lon. This protein also participates in negative autoregulation of expression of the operon of the *phd-doc* module by binding to the operator sequences of the operon promoter in several regions. On multiplication of operon copies in the cell, the operon expression is suppressed; thus, negative autoregulation is important for the mechanism of plasmid maintenance by the cell.

The *mazEF* module is a region of *E. coli* chromosomal locus responsible for the synthesis of guanosine-5',3'-bispyrophosphate (ppGpp) effector molecule under acute amino acid deficiency in a cell. The level of expression of this locus is strictly regulated by ppGpp concentration. The properties of the *mazEF* module suggest a model of apoptosis in *E. coli*. Under amino acid starvation, the level of ppGpp in the cell increases and, as a result, the synthesis of both the labile antitoxin protein MazE and the stable toxin MazF is suppressed. The concentration of labile antitoxin in the cell decreases due to the function of specific proteases and the toxic protein MazF affects a number of cell targets, resulting in cell death. Thus, the *mazEF* module is regulated by low-molecular signal metabolites.

Previously we have constructed a series of linear vectors carrying the genes of phage P1 segregation stability system.

The first two vectors, pP1lin1 and pP1lin2, are based on the P1 replicon and additionally contain the genetic elements of phage N15 (protelomerase and pin telomeres) providing replication of the vector as a linear form. Linear vector pG594, on the contrary, is based on the linear replicon of phage N15. It contains the replicon and the gene of telomere-forming enzyme of phage N15 along with the *par* module of segregational stability of phage P1. The circular plasmid pZC176 was used as a control. This plasmid carries a DNA fragment of phage P1 (about 6 kbp) containing the determinants of replication and segregational stability of prophage P1 and the genes providing resistance to ampicillin (*bla*) and spectinomycin (*aad*).

The objective of this stage of the work was to determine the levels of stability of inheritance of these vectors under nonselective conditions with the purpose of choosing the optimal basal linear vector for subsequent integration of toxin–antitoxin genetic modules.

In order to determine the level of stability of inheritance of the vectors pP1lin1, pP1lin2, and pG594 under nonselective conditions, we introduced them into the strain DH10B; bacterial cultures were grown up to the stationary phase in LB medium with selective antibiotics. Then a fresh culture was diluted 1000 times and grown in LB broth without antibiotics until the stationary phase, which corresponded to ten generations. Then, the cells were inoculated onto plates without the antibiotic and replicas were made onto plates with the antibiotic; growth was possible for plasmid-containing colonies only. The experiment was repeated in the course of 40 generations in the absence of the antibiotic.

It was established that the vectors pP1lin1 and pP1lin2 were unstable and quickly lost under nonselective conditions (the rate of loss was 6–8% per generation). At the same time, the linear vector pG594 based on the N15 replicon with the *par* operon of phage P1 proved to be stable: the rate of its loss did not exceed 0.01% per generation, corresponding to the data on the control circular plasmid pZC176. Thus, the *par* operon of P1 can ensure stable inheritance of a linear plasmid (pG594) in *E. coli*. Rather than a defect in the functioning of the segregation stability system, the low efficiency of their DNA replication may be an explanation for the instability of pP1lin1 and pP1lin2, since it possibly results in the presence of less than two plasmid copies in a cell at the moment of cell division, which automatically leads to formation of a plasmid-free daughter cell after division.

Thus, the most promising object for construction of stable linear expression vectors is plasmid pG594. The potential sources of plasmids with addiction modules may be natural isolates of enterobacteria from the feces of warm-blooded animals. Previously, we have shown that the microbial community of horse intestines simultaneously contains, on average, about 1000 genetically different strains of coliform microbes

Numbers of colonies obtained after inoculation of identical dilutions of the cultures containing plasmids with the *ccd* and *phd-doc* type modules in the presence of an incompatible plasmid

Type of insert	Colony number, CFU/ml			
	Inoculation without antibiotics	Kanamycin and ampicillin selection	Ampicillin selection	Kanamycin selection
Ccd	680	257	433	270
Phd-doc	927	402	372	180
Control	854	366	220	314

Note: The plasmid carrying a neutral insert corresponding in size to the modules under study was used as a control.

evolving very rapidly under the selective pressure of bacteriophages [13].

The design of universal oligonucleotides for amplification of the modules was particularly difficult because of the extraordinarily high G + C content in the sequences of the modules proper and in their immediate environment. However, the developed original primers proved very useful for our work, giving highly specific amplification and high yields of the target PCR product.

One of the factors considerably impeding PCR directly on the templates (the aqueous extracts from horse feces or wastewater bacteria concentrated by centrifugation) is a substantial content of the substances partially or completely inhibiting the *Taq* polymerase in this biological material. For preventing difficulties with PCR inhibition, the bacteria were isolated by culture methods, plasmid DNA preparations were obtained, and cell lysates containing total DNA suitable for PCR amplification were prepared.

Out of the 50 analyzed samples, 25 were animal feces and the other 25 were wastewater samples. The titers of coliform bacteria were $2 \times 10^5 - 5 \times 10^6$ CFU/g and 10^3–10^5 CFU/g for the fecal material and wastewater samples, respectively.

Four separate isolates from each sample were analyzed (100 in all). Plasmid DNA isolation showed that 23 and 11 isolates from feces and wastewater, respectively, contained plasmids of 5–20 kbp.

Samples of the relevant plasmid DNA were kept for further study. However, the total preparations of plasmid DNA were prepared from washouts of a large number of colonies to cover more completely the genetic diversity of natural plasmids.

The colonies of coliform microflora (300–1000) grown on a solid medium without competing with each other for the nutrient substrate were washed off the plates with normal saline solution, possible sodium lauryl sulfate admixtures were washed off in three successions of the saline solution, and the biomass was divided into three portions.

The first portion was deposited into the collection of microorganisms stored at –70°C; plasmid DNA was isolated from the second portion by the standard method of phenol lysis; and the third portion was exposed to heat shock (100°C), centrifuged, and dilutions of its aqueous extract were used directly as templates in PCR with the universal primers.

The optimized polymerase chain reaction with the developed oligonucleotide primers (see above) hybridized with the DNA sequences of desired loci yielded PCR products carrying the sequences of all three sought addiction modules. It would be interesting to mention that all three of these modules were found only in the two DNA samples isolated from wastewater microbes, whereas PCR of nearly all DNA samples from microbes of the horse intestines community resulted in stable specific amplification of the target products with high yields.

The obtained PCR products with the sought modules were used for making clone libraries in the pGEM-T vector (Promega). The sequence of inserts was analyzed by sequencing from the standard primers M13F and M13R. Two clones of each type were sequenced altogether. Analysis of the sequences confirmed that the inserts contained the sequences of toxin–antitoxin modules close but not identical to the previously published sequences of the *phd-doc, mazEF*, and *ccd* cassettes. Three clones with the sought modules were selected for further testing of plasmid inheritance.

Thus, natural populations of enterobacteria were successfully screened for the presence of plasmids bearing different types of modules that caused postsegregational cell death of plasmid-free variants.

The nucleotide sequences of inserts of the three previously selected clones were identified as described in Materials and Methods. As a result, it was confirmed that all three clones under study contained toxin–antitoxin modules. In two cases, these modules proved to be close (but not identical) to the *ccd* module, and in one case the module was of the *phd-doc* type (GenBank #GQ398086.1, AF234173.1).

Stability of the plasmids carrying toxin–antitoxin modules of the *ccd* and *phd-doc* types was comparatively analyzed in a two-plasmid model system described in Materials and Methods. Results of the analysis are presented in the table.

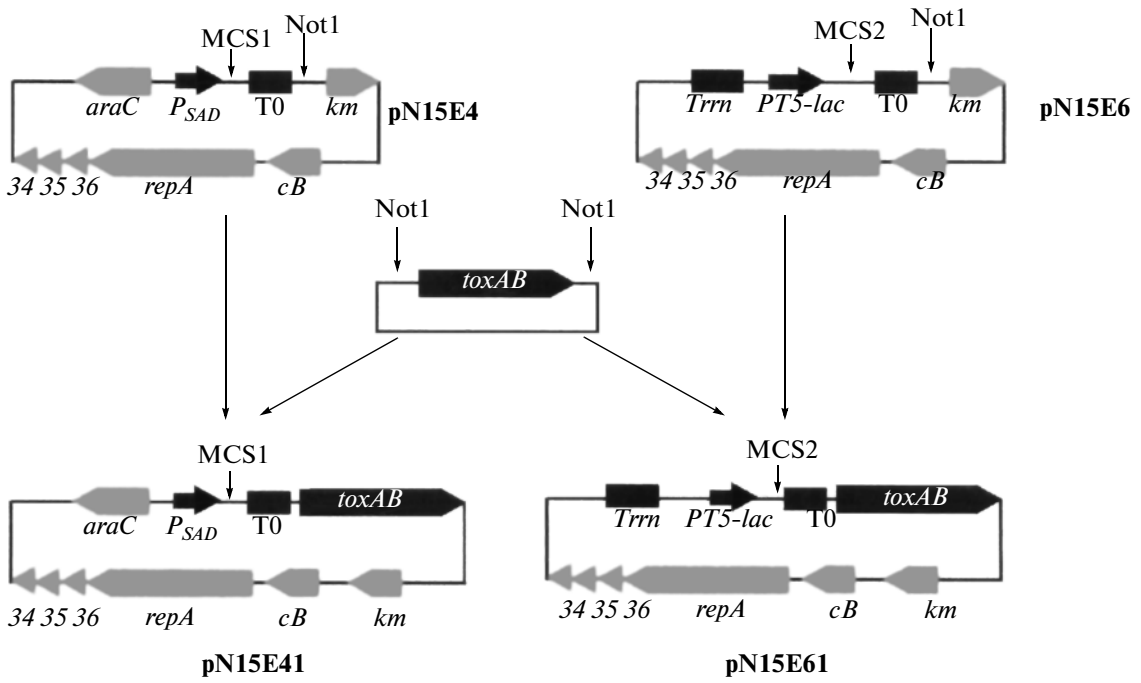

Fig. 1. The scheme of construction and the structures of expression vectors. The designations are interpreted in the text.

Thus, the *ccd* module most effectively prevents the loss of its carrying vector under competition with a non-compatible plasmid.

Previously, two circular expression vectors, pN15E4 and pN15E6 were constructed, based on the phage N15 replicon and *E. coli* strains for their maintenance. The characteristic property of these expression systems was the possibility of regulating product synthesis on two levels: through controllable variation of the number of vector copies and through activation of the induced promoter. Both vectors were circular low-copy plasmids carrying a *cis*-acting component (centromere) of the segregation stability system of phage N15 but not the genes of the *sop* operon, the products of which provided distribution of the plasmids with centromeres in the daughter cells before partition. Thus, the vectors pN15E4 and pN15E6 are instable in the standard *E. coli* strains under nonselective conditions. However, in the constructed strain DH31 *sop lacI* containing the chromosome-integrated copy of the N15 *sop* operon, both plasmids are stably inherited owing to the segregation stability mechanism located in the plasmids (active *in cis* centromeres) and in the chromosome (the *sop* operon with active *in trans* products).

The objective of this stage of our work was construction of expression vectors based on pN15E4 and pN15E6 and containing additionally a toxin–antitoxin genetic module.

The vector pN15E4 contained the following genetic elements: the *araC* regulatory gene, the *araP-bad* promoter, a polylinker (the site of target gene cloning), the T0 terminator of phage lambda, the gene of kanamycin resistance, and the components of N15 phage replicon (genes 38–34). The vector pN15E6 contained a hybrid PT5-*lac* promoter instead of *araP-bad* and an additional transcription terminator TrrnB (four copies) in the place of *araC* (Fig. 1). The unique restriction site NotI located between the T0 terminator and the gene of kanamycin resistance was chosen as a place of insertion of the toxin–antitoxin module. Being located in this region of the vector, the toxin/antitoxin module will be protected by the T0 terminator from transcription initiated from the induced promoter of the vector, which must exclude unwanted overexpression of the toxin component of the module.

For the construction of vectors with the toxin–antitoxin module, the DNA fragment corresponding to the *ccd* module was cut out of the previously selected plasmid using the NotI restriction endonuclease and cloned into the NotI site of the vectors pN15E4 and pN15E6. The recombinants were selected, where the orientation of the insert coincided with the orientation of the promoter. As a result, expression vectors pN15E41 and pN15E61 were constructed (Fig. 1).

The vectors pN15E41 and pN15E61 and the initial vectors pN15E4 and pN15E6 were introduced into the strain DH10B in order to determine the level of stability of their inheritance under nonselective conditions; bacterial cultures were grown until the stationary

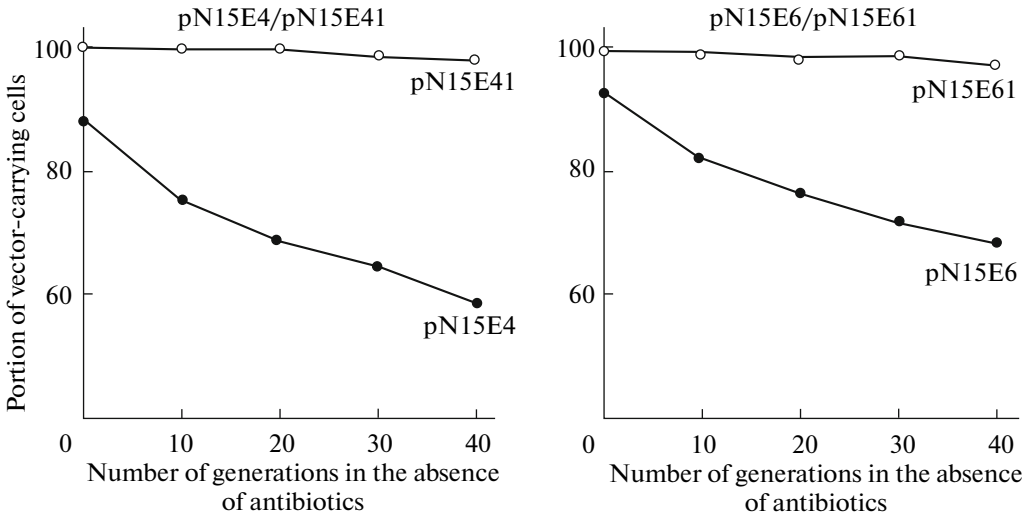

Fig. 2. Inheritance of vectors pN15E4, pN15E41, pN15E6, and pN15E61 in the strain DH10B during culture growth in the absence of antibiotic.

phase in LB with the selective antibiotics. Then the fresh culture was diluted 1000 times and grown in LB broth without antibiotics until the stationary phase, which corresponded to ten generations. The cells were inoculated onto plates without antibiotics and then the replicas were made onto plates with the antibiotic; growth was observed for plasmid-containing colonies only. The experiment was repeated during 40 generations in the absence of antibiotics.

As expected, vectors pN15E4 and pN15E6 were instable in the strain DH10B not containing the *sop* operon of N15. At the same time, their derivatives containing the toxin–antitoxin modules, pN15E41 and pN15E61, proved to be much more stable; the observed frequency of their loss did not exceed 0.1% per generation (Fig. 2). Thus, the new toxin–antitoxin module is really active in *E. coli* cells and can be used for plasmid stabilization.

Stability of the vectors pN15E41 and pN15E61 was also characterized in the strain DH31 *sop lacI* containing the chromosome-integrated *sop* operon of phage N15. In this case, both mechanisms of plasmid stabilization, the system of segregational stability and the toxin–antitoxin module, must act concurrently. Stable maintenance of the vectors pN15E41 and pN15E61 in the strain DH31 *sop lacI* (plasmid-free cells were not detected), the synergism of action of both systems, has been demonstrated.

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