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EXPERIMENTAL ARTICLES

Stability of Recombinant Plasmids in Transgenic Microorganisms under Different Environmental Conditions

L. Yu. Popova, E. E. Maksimova[1], T. I. Lobova, T. V. Kargatova, A. N. Boyandin, T. Yu. Krylova, and N. S. Pechurkin

Institute of Biophysics, Siberian Division, Russian Academy of Sciences, Krasnoyarsk, 660036 Russia

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Abstract—The copy number of R plasmids weakly depends on the selective pressure of the respective antibiotic but does depend on the physiology of the host species and the type of plasmids and cloned genes, whose expression leads to a further load on the biosynthetic apparatus of cells. The last factor is critical in the maintenance of recombinant plasmids in transgenic microorganisms.

Key words: recombinant plasmids, transgenic microorganisms, antibiotic resistance, bioluminescence, interferon.

The phenomenon of spreading and stabilization of R plasmids in natural bacterial populations needs extensive investigation because of a wide application of R plasmid–based vectors for the construction of transgenic microorganisms. To provide for plasmid stability in cells, microorganisms containing R plasmids are commonly maintained using media supplemented with respective antibiotics. There is a controversy in the literature as to the stability of R plasmids during the multiple successive transfer of recombinant microorganisms without antibiotics. Some authors reported on the stability of plasmids subjected to such transfer [1, 2], whereas others reported on a decrease in their number [2–4]. Of great theoretical and practical interest is the spread of drug resistance plasmids and the appearance of multiple drug resistance in bacterial populations. It was found that such a resistance is due to conjugative plasmids carrying a cassette of antibiotic resistance genes [1]. Nonpathogenic *Escherichia coli* strains living in the intestines of vertebrates may serve as intermediate hosts that transfer R plasmids to pathogenic and saprophytic bacteria through the so-called horizontal gene transfer [4, 5].

The size of R plasmids varies widely [6, 7]. The construction of a large number of recombinant plasmids based on R plasmids may result in an uncontrolled spread of antibiotic resistance in nature. Taking into account an insignificant concentration of antibiotics in the environment, it can be suggested that the main factors promoting the maintenance of plasmids in natural bacterial populations are the properties of host cells and specific environmental conditions.

[1]Corresponding author. E-mail: lena@ibp.ru.

The aim of this work was to study the stability of R plasmids in transgenic microorganisms under different environmental conditions.

MATERIALS AND METHODS

Bacterial strains. Experiments were carried out with *Escherichia coli* Z905/pUC18 [8], *E. coli* Z905/pPHL7 [9, 15], *Bacillus subtilis* 2335/pBMB105 [10], and clones of transgenic microorganisms introduced into model aquatic microcosms and isolated from them at different times after introduction.

Plasmids studied in this work were as follows: Plasmid pUC18 (Ap^r) had a size of 2.7-kbp. Plasmid pPHL7 (Ap^rLux^+) 12.4-kbp in size was constructed on the basis of vector pUC18 (Ap^r) and contained the *lux* operon of the luminescent marine bacterium *Photobacterium leiognathi* cloned under the control of the *lac* promoter [11]. Plasmid pBMB105 (Km^rInf^+) 5.6-kbp in size was constructed on the basis of vector pBMB105 (Km^r) and contained the human α-2 interferon gene cloned under the control of the constitutive α-amylase promoter.

Cultivation media. Minimal essential M9 medium for the cultivation of microorganisms contained (g/l) Na_2HPO_4, 6; KH_2PO_4, 3; NaCl, 3; and NH_4Cl, 1. Solutions of $MgSO_4$ (20%), $CaCl_2$ (0.5%), and glucose (or glycerol) (20%) were sterilized separately and added to a sterile M9 medium in amounts of 1, 1, and 10 ml, respectively [12]. The medium was also supplemented with 5 g/l peptone. Solid media were prepared by adding agar in an amount of 20 g/l. The concentration of ampicillin in the medium varied from 0.5 to 50 μg/ml and that of kanamycin varied from 0.5 to 1000 μg/ml.

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Strains, plasmids, and cultivation conditions used in experiments on the evaluation of the stability of R plasmids in transgenic *Escherichia coli* and *Bacillus subtilis* bacteria

Plasmid	Strain	Cultivation conditions	Selective factor	Ref.
pUC18	*E. coli* Z905	Chemostat in M9 medium	Ampicillin	[8]
			–	[8]
pPHL7	*E. coli* Z905	Chemostat in M9 medium	Ampicillin	[8]
			–	[8]
		Subculturing in M9 medium with peptone	Ampicillin	[15]
			–	[15]
		Subculturing in M9 medium without peptone	Ampicillin	[15]
			–	[15]
		Introduction into microcosms	–	[9, 13]
pPHL7	*E. coli* Z905-2	Subculturing on agar M9 medium without peptone	Ampicillin	[15]
			–	[15]
		Subculturing in M9 medium with peptone	Ampicillin	[15]
			–	[15]
pPHL7	*E. coli* Z905-3	Subculturing in M9 medium without peptone	Ampicillin	This work
			–	This work
		Subculturing in M9 medium with peptone	Ampicillin	This work
		Subculturing in M9 medium without peptone	–	This work
pBMB105	*B. subtilis*	Subculturing on agar M9 medium without peptone	Kanamycin	[10, 14]
			–	[10, 14]
		Subculturing on agar M9 medium with peptone	Kanamycin	[10, 14]
			–	[10, 14]
		Introduction into microcosms	–	[10, 14]

Cultivation conditions are listed in the table. Strains were grown in liquid and on solid media in the presence or absence of selective factors [10]. Batch cultures were grown at 28°C in 250-ml flasks with 50 ml of the growth medium on a shaker. Continuous cultivations were carried out in a 300-ml fermentor operated in a chemostat mode at a dilution rate of 0.1 h^{-1} [8]. The fermentor was inoculated with overnight batch cultures grown in a chemically defined medium with glucose and ampicillin. Cultures were stirred at a rate of 600 rpm. Air was pumped at a rate of 1 l/min. The cultivation temperature was controlled at a level of 30 ± 1°C. The pH of the chemostat culture was controlled within 7.0–7.5. The nutrient medium was fed into the fermentor using a peristaltic pump.

Bacterial cells with small and large numbers of plasmid copies were distinguished by the intensity of their growth on complete solid media in the presence of respective antibiotics added at a concentration of 50 mg/l. Plasmid DNA was isolated from exponential-phase cells by the standard procedure [13, 14]. Before electrophoresis, samples of plasmid DNA were dissolved in the electrophoresis buffer aliquots, whose volume was proportional to the biomass from which a given DNA sample was isolated. The concentration of plasmid DNA was evaluated using the Scion Image program for Windows.

The adaptation capacity of transgenic microorganisms (TMs) was studied using model aquatic microcosms [14]. Bacterial strains were introduced into the microcosms in a way similar to their inoculation to the chemostat. Cells for inoculation were precipitated from an overnight culture by centrifugation at 8000 *g* for 30 min and washed off the nutrient medium with sterile tap water.

The optical density of bacterial suspensions was measured at 540 nm using a KFK-2 spectrophotometer. The luminescence intensity of *E. coli* Z905/pPHL7 clones was measured using a bioluminometer manufactured at SKTB Nauka, Krasnoyarsk.

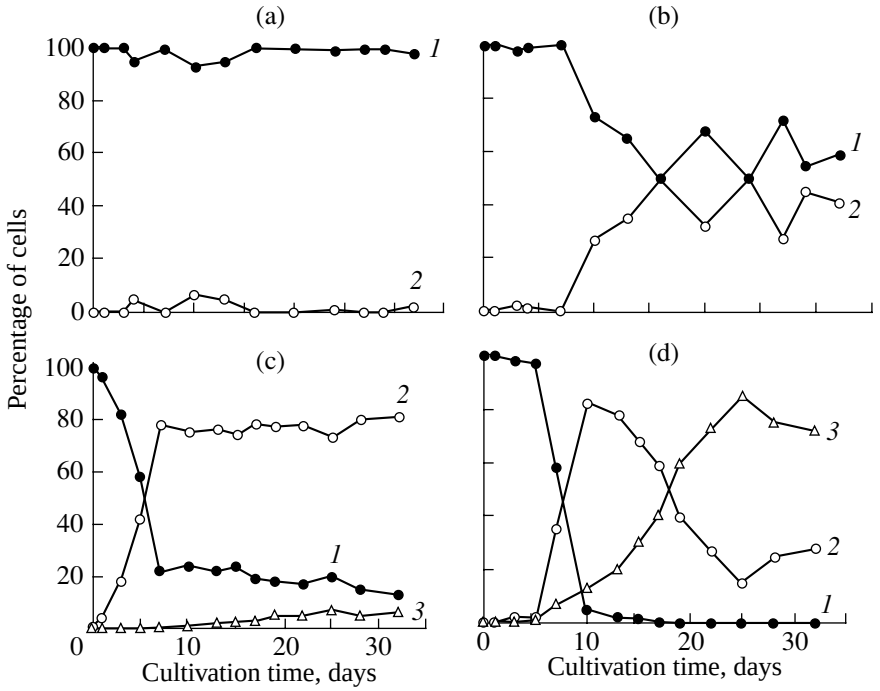

Fig. 1. The structure of the population of the recombinant (a, b) *E. coli* Z905/pUC18 and (c, d) *E. coli* Z905/pPHL7 strains grown in chemostat cultures in the (a, c) presence and (b, d) absence of ampicillin: (*1*) cells with the original number of plasmid copies (and the original level of expression of the *lux* operon in the *E. coli* Z905/pPHL7 culture); (*2*) cells with a low number of plasmid copies; and (*3*) cells with the superexpression of the *lux* operon.

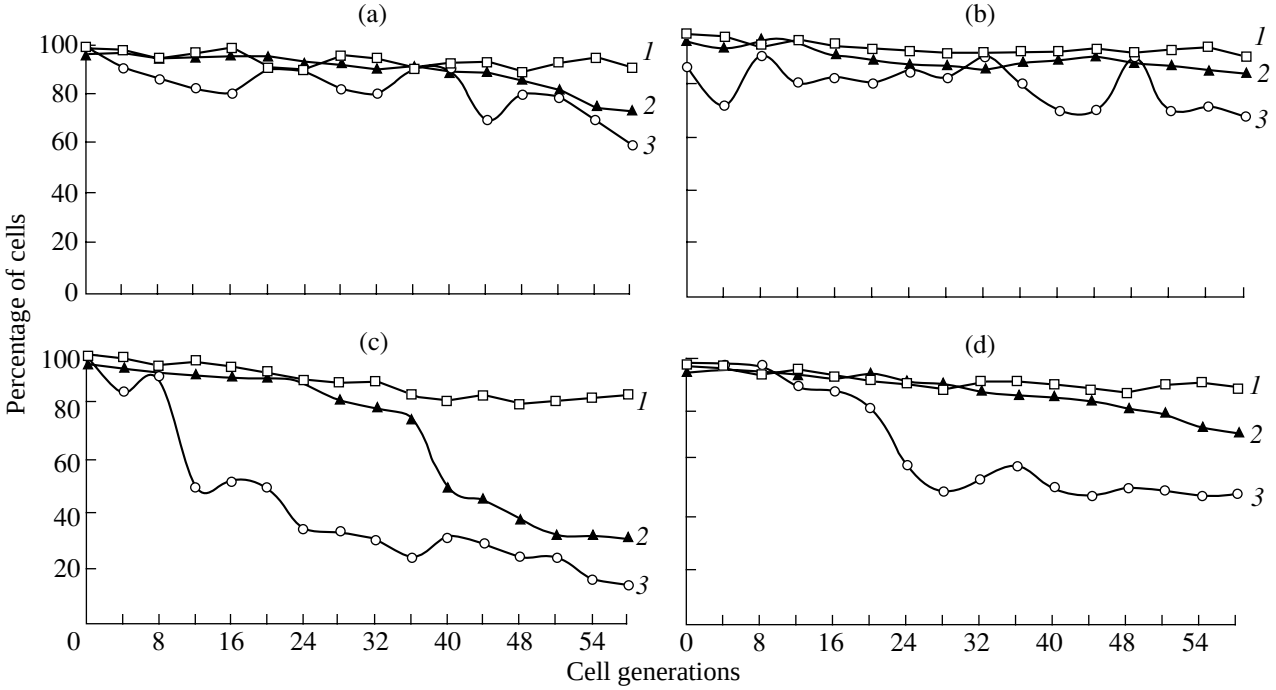

Fig. 2. The percentage of cells with the original copy number of the recombinant plasmid pPHL7 in the populations of (*1*) *E. coli* Z905/pPHL7, (*2*) *E. coli* Z905-2/pPHL7, and (*3*) *E. coli* Z905-3/pPHL7 strains grown in (a) M9 medium with peptone in the absence of ampicillin, (b) M9 medium with peptone in the presence of 50 mg/l ampicillin, (c) M9 medium without ampicillin, and (d) M9 medium with 50 mg/l ampicillin.

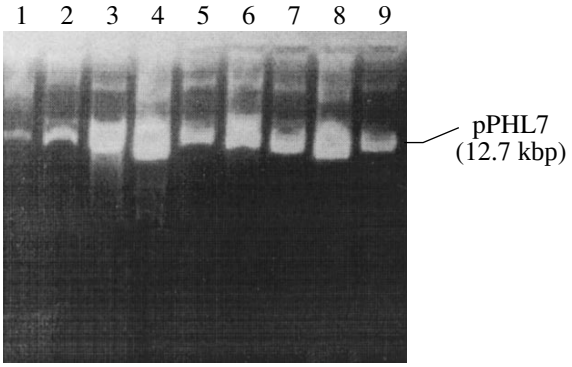

Fig. 3. Electrophoresis of the plasmid DNA of clones isolated after the subculturing of the *E. coli* Z905/pPHL7 strain in M9 medium with peptone (clones 11-43, 11-143, and 11-161), M9 medium with peptone and ampicillin (clones 21-218 and 21-31), M9 medium (clone 31-18), and M9 medium with ampicillin (clones 41-328 and 41-324). Lanes: (1) clone 41-328 (39.8%); (2) clone 21-218 (53.2%); (3) clone 31-31 (70.8%); (4) control (plasmid pPHL7) (100%); (5) clone 11-143 (66.1%); (6) clone 31-18 (78.4%); (7) clone 11-161 (84%); (8) clone 11-43 (74.9%); and (9) clone 41-174 (35.9%). Parenthesized are the relative numbers of plasmid copies with respect to the control.

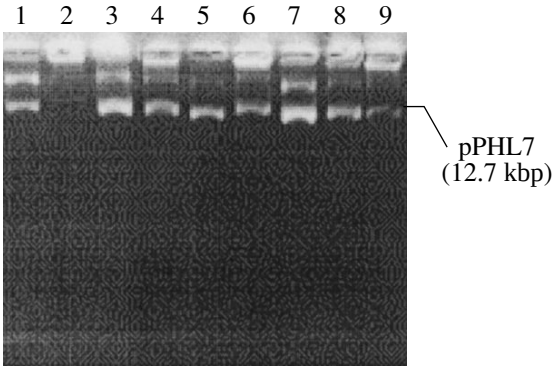

Fig. 4. Electrophoresis of the plasmid DNA of clones isolated after the subculturing of the *E. coli* Z905-2/pPHL7 strain in M9 medium with peptone (clone 12-306), M9 medium with peptone and ampicillin (clones 22-327, 22-51, and 22-118), M9 medium (clone 32-450), and M9 medium with ampicillin (clones 42-2, 42-351, and 42-447). Lanes: (1) clone 42-2 (66.6%); (2) clone 42-351 (44.1%); (3) control (plasmid pPHL7) (100%); (4) clone 32-450 (50%); (5) clone 12-306 (58.3%); (6) clone 22-327 (48.3%); (7) clone 22-51 (91.6%); (8) clone 22-118 (48.3%); and (9) clone 42-447 (45%). Parenthesized are the relative numbers of plasmid copies with respect to the control.

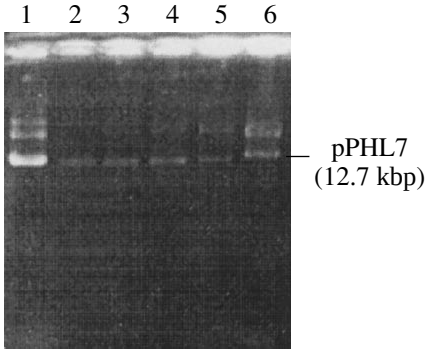

Fig. 5. Electrophoresis of the plasmid DNA of clones isolated 14 days (clone III-6-3), 246 days (clones I-27, I-43, and II-58), and 540 days (clone II-95) after the introduction of the *E. coli* Z905/pPHL7 strain into the model aquatic microcosm. Lanes: (1) control (plasmid pPHL7) (100%); (2) clone I-27 (46.3%); (3) clone III-6-3 (44.8%); (4) clone I-43 (48.1%); (5) clone II-58 (41.1%); and (6) clone II-95 (63.7%). Parenthesized are the relative numbers of plasmid copies with respect to the control.

RESULTS AND DISCUSSION

The stability of plasmid pUC18 was investigated in a chemostat culture of *E. coli* Z905/pUC18 [8]. During cultivation in the presence of ampicillin, nearly 100% of the cells retained the initial number of plasmid copies. In the absence of ampicillin, some cells, whose relative number reached 30% after 10 days of cultivation, had a reduced number of plasmid copies (Figs. 1a and 1b).

The stability of plasmid pPHL7 in a chemostat culture of *E. coli* Z905/pPHL7 was poor even under selective conditions, i.e., in the presence of ampicillin (Fig. 1). The fraction of cells with a low number of plasmids reached 85%. Since plasmid pPHL7 was constructed on the basis of plasmid pUC18 [8], which is stable under selective conditions, the instability of plasmid pPHL7 can be explained by the fact that it contains a fragment of the *lux* operon, whose expression leads to a further load on the biosynthetic apparatus of cells and thus may reduce the copy number of the plasmid. The decline in the ampicillin resistance of cells correlated with a decline in their luminescence.

It was found that cells with low copy numbers of plasmid pPHL7 were not washed out from the chemostat even in the presence of ampicillin in the nutrient medium. This was probably due to the degradation of this antibiotic by the cells that have large numbers of plasmid pPHL7 copies (Fig. 1c). In the chemostat culture without ampicillin, when the synthesis of β-lactamase is not necessary for cell survival, the number of cells with the initial number of the plasmid pPHL7 copies and superexpression of the *lux* operon tended to increase (Fig. 1d). Such cells were designated as *E. coli* Z905-3/pPHL7 (see table). Our earlier studies showed that the weak luminescence of recombinant cells can be related to the repression of the expression of luminescence genes [15]. In general, plasmid pPHL7 was relatively stable in bacterial cells.

In liquid M9 medium, the stability of plasmid pPHL7 was better in the presence of peptone. In M9 medium without ampicillin, the content of cells with

low copy numbers of plasmid pPHL7 was maximum (see table and Figs. 2a, 2b, and 3). In all cultivation media, the fraction of cells of the *E. coli* Z905-2/pPHL7 strain (recall that this strain is characterized by the constitutive expression of the cloned *E. coli* genes) with low copy numbers of plasmid pPHL7 was larger than the fraction of such cells with the inducible synthesis of the *lux* operon (Figs. 2c, 2d, and 4). It is obvious that the reduction of the number of plasmid copies in *E. coli* Z905-2/pPHL7 cells is the only way to diminish the expression of the cloned *lux* genes. In the population of *E. coli* Z905-3/pPHL7 cells with the superexpression of the *lux* operon, the fraction of low-plasmid-number cells did not exceed 10% under all experimental conditions (Fig. 2).

Ten days after the introduction of the *E. coli* Z905/pPHL7 strain into model microcosms, we were unable to detect cells of this strain with the original number of plasmid copies (Fig. 6c). *E. coli* Z905/pPHL7 cells with a low number of plasmid copies lost their resistance to ampicillin and showed a level of the *lux* operon expression that was 2 to 6 orders of magnitude lower than in the case of cells with a normal number of plasmid copies. One year after the introduction, some clones retained 50–80% of the initial copy number of plasmid pPHL7 and showed a 4 to 5 orders of magnitude lower intensity of luminescence than the original cells (Fig. 6). Most of the cells with a low number of plasmid copies remained luminescent only in the presence of high concentrations of ampicillin in the medium (Fig. 7). The growth rate of such cells was low, suggesting the induction of the expression of plasmid genes. The original cells exhibited such induction only in a M9 medium with peptone (Fig. 7). Clones with the suppressed expression of plasmid genes and an original or decreased number of plasmid copies actively grew in media with high concentrations of ampicillin (Fig. 7).

It should be noted that one day after the introduction of the *E. coli* Z905/pPHL7 strain into the microcosms, some cells of this strain exhibited the constitutive synthesis of the luminescence system (Fig. 6) against the background of a low number of copies of the recombinant plasmid. Such cells could not efficiently compete with the indigenous microflora of the microcosms, so that, soon after the introduction, the population of the introduced cells was dominated by those which had a suppressed expression of the cloned *lux* genes. The number of plasmid copies in the latter cells gradually returned to the original value (Figs. 5 and 6). Therefore, the introduced *E. coli* Z905/pPHL7 cells retained their recombinant plasmid irrespective of the composition of the nutrient medium and the presence of ampicillin.

The strain *B. subtilis* 2335/pBMB105 grown in a M9 medium with peptone and kanamycin showed a poorer stability of plasmid pBMB105 than in the same medium without peptone (see table). When introduced into the microcosms, cells of this strain produced spores. The vegetative cells grown from those spores

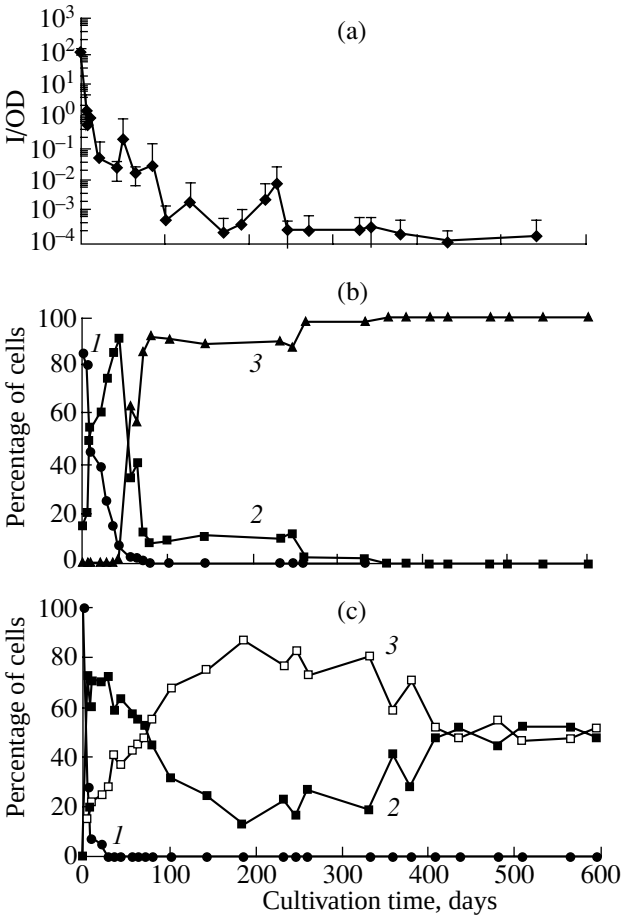

Fig. 6. Dynamics of the expression of the cloned *lux* genes in the *E. coli* Z905/pPHL7 cells introduced into the model aquatic microcosm. (a) Specific luminescence of cells; (b) percentage of cells with (*1*) initial, (*2*) weak, and (*3*) intense expression of the *lux* genes; and (c) percentage of cells with (*1*) initial (~100 plasmid copies per cell), (*2*) twofold reduced, and (*3*) more than twofold reduced plasmid copy number.

had the original number of plasmid copies. Some vegetative cells of this strain isolated from the microcosms showed a reduced number of plasmid copies [10, 14]. Commonly, the reduction in the plasmid copy number correlated with the cell resistance to kanamycin. For instance, the *B. subtilis* 2335 10-44/pBMB105 clone had a better kanamycin resistance (1000 mg/l) (Fig. 9) and more plasmid copies than the introduced parent strain. On the other hand, the *B. subtilis* 2335 I-15/pBMB105 clone, which was also resistant to 1000 mg/l kanamycin, had the same number of plasmid copies as the parent strain (Fig. 8). This discrepancy can be explained by different levels of expression of the cloned α2-interferon genes and, therefore, different loads on the biosynthetic apparatus of cells.

The behavior patterns of the recombinant plasmids pPHL7 and pBMB105 in bacterial cells were different.

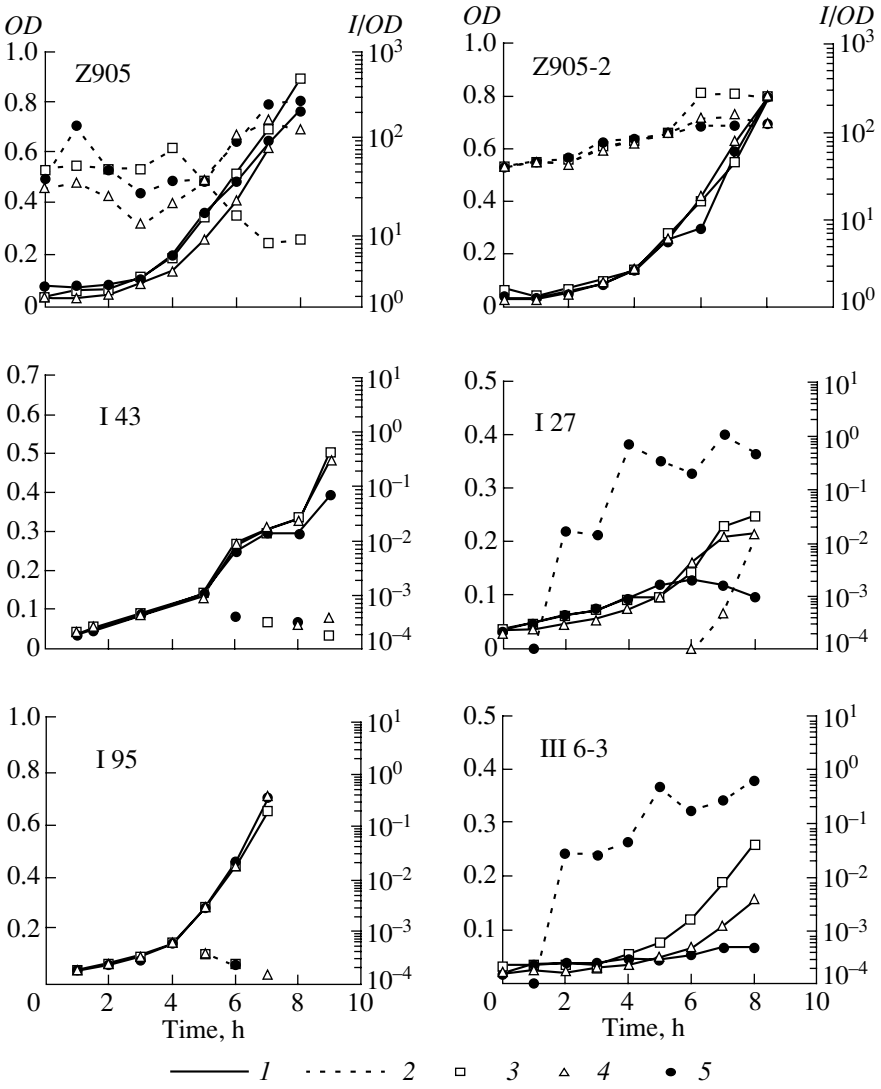

Fig. 7. Dynamics of the (*1*) growth and (*2*) luminescence of some *E. coli* Z905/pPHL7 clones isolated from the microcosm some time after introduction and cultivated in M9 medium with peptone and (*3*) 0, (*4*) 0.5, and (*5*) 50 mg/l ampicillin.

The population of *E. coli* Z905/pPHL7 cells actively growing in a chemostat culture, where each of the retained plasmid copies considerably affects the culture growth, was dominated by cells with a low number of plasmid copies and a low intensity of luminescence. The fraction of such cells was particularly large under selective conditions (in the presence of ampicillin). Cells with a great number of plasmid copies were also rapidly eliminated from the model microcosms, where the growth rate of cells was low.

The recombinant *B. subtilis* 2335/pBMB105 cells rapidly lost their plasmids even in the presence of kanamycin, which can be accounted for by the fact that the heterologous genes were cloned under the constitutive promoter and, therefore, can diminish the expression of the cloned genes only by reducing the number of the recombinant plasmid copies. The recombinant *E. coli* Z905/pPHL7 strain can efficiently be used for the evaluation of the effect of various environmental factors on the stability of recombinant plasmids and the expression of cloned genes, as the decrease in the number of plasmid copies correlates with the degree of expression of the cloned genes. Transgenic microorganisms with the cloned genes of bacterial luminescence are particularly promising for this purpose, since the expression of such genes can easily be monitored by measuring cell luminescence.

In our experiments, recombinant cells were never observed to lose their plasmids. Even if some *B. subtilis* 2335/pBMB105 clones happened to lose them, such clones restored their plasmids during the subsequent subculturing on media with increasing concentrations of kanamycin (Fig. 8).

To conclude, under certain conditions dependent on the microorganism, environment, plasmid construction, and the load of plasmid expression on the biosynthetic apparatus of cells, the role of selective factors (i.e., anti-

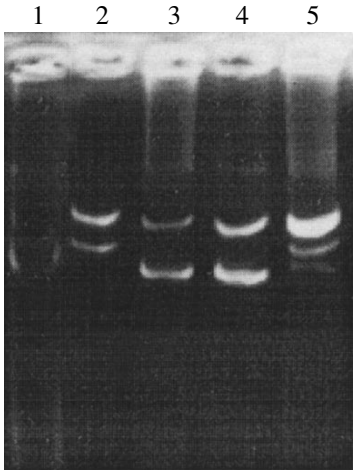

Fig. 8. Electrophoresis of the plasmid DNA isolated from (1) *B. subtilis* 2335 10-111/pBMB105 (41%), (2) *B. subtilis* 2335 5-54/pBMB105 (73%); (3) *B. subtilis* 2335 I-15/pBMB105 (90%); (4) *B. subtilis* 2335/pBMB105 (control, 100%); and (5) *B. subtilis* 2335 10-44/pBMB105 (119%). Parenthesized are the relative numbers of plasmid copies with respect to the control.

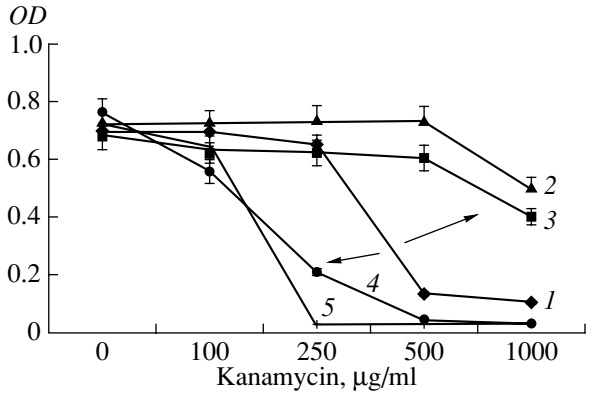

Fig. 9. Susceptibility to kanamycin of some *B. subtilis* 2335/pBMB105 clones: (*1*) clone 2335 (parent); (*2*) clone 2335 I-15 (isolated from the aquatic microcosm 75 days after introduction); (*3*) clone 2335 10-44 (isolated after 10 subcultures in M9 medium with peptone and kanamycin); (*4*) clone 2335 5-54 (isolated after 5 subcultures in M9 medium); and (*5*) clone 2335 10-111 (isolated after 10 subcultures in M9 medium with kanamycin).

biotics) in the stability of plasmids in recombinant microorganisms may be insignificant.

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