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LONG-TERM PRESERVATION OF UNSTABLE BACTERIOPHAGES OF ENTEROBACTERIA

Bacteriophages are integral components of bacterial communities. Their practical applications and significance for humans are various. Thus, keeping phage collections along with their specific host bacteria is an urgent and important mission for biologists. The problems of the long-term storage of phages steel are not completely solved. The main difficulties may occur due to the structural instability of virions as well as an accelerated genetic variability in phage genomes both in vivo and in vitro. In the paper the results of 10-years observation over unstable bacteriophage storage process was presented as well the method of their long-term preservation was proposed. It consisted of the optimization of STMG buffer system (200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 100 μg/ml gelatin) [Serwer P., Pichler M.E. Electrophoresis of bacteroiphage T7 capsids in agarose gels// J.Virol. – 1978. – V.28, N3. – P.917-928] by higher gelatin concentration or its replacement by ficoll (2 – 6 %), and increasing of Mg^{2+} concentration to 10 mM. The proposed buffer composition allowed saving structural entirety of unstable phage virions during their long-term storage.

K e y w o r d s: bacteriophages, long-term storage, structural instability, gelatine, ficoll.

Recent achievements in molecular microbiology and genomics made it possible considering bacteriophages as integral components of bacterial communities [7] that carry out the horizontal gene transfer [15] and increase the adaptive capability of bacteria. There are many aspects in their practical applications. For example, bacteriophages historically serve as model objects in fundamental biological research [20] and may be used as molecular tools for studies of proteins [12], vectors in gene engineering [4] and tools in bacterial genetics [2], in detection and identification of pathogenic bacteria [16], as templates in interdisciplinary nanotechnologies and self-sufficient nanostructures [6, 13]. Moreover, bacteriophages reasonably are going behind as potential immunomodulators [5] as well as antimicrobial agents [10] of the upcoming age – the medicine without antibiotics, and now they are used in therapy of bacterial infections in humans, animals, fisheries and agrobusiness. For all these reasons, keeping collections of bacteriophages along with their host bacteria is an urgent and important mission for biologists.

The problems of the long-term storage of phages still are not completely solved. The main difficulties may occur due to the structural instability of the virions as well as the accelerated genetic variability in phage genomes both *in vivo* and *in vitro* [3].

Bacteriophage ZF40 [8] of A1-morphotype is one of the rare phages of important phytopathogenic bacterium *Pectobacterium carotovorum* subsp. *carotovorum* which is one of the main objects in our lab during last decades. Previous studies have shown that the phage is very sensitive to sharp variations in the environment. Therefore, the main goal of the present work was an optimization of the conditions for long-term preservation of unstable enterobacterial phages which have structural instability similar to that of phage ZF40.

Materials and Methods

Erwiniophage ZF40 *P. carotovorum* subsp. *carotovorum* (Pcc) was the main object of the present research. The mutant strain Pcc RC5297 [18] served as the host bacterium for the phage ZF40. This phage, as well as polyvalent bacteriophage FE 44 [17] and erwiniophage 49 [19] were obtained by the confluent lysis method [11]. Gelatin or ficoll were added to the phage lysates to the final concentrations 100 μg/ml and 2%, respectively. It was growing on agar containing nutrient media LB and A [11] with lactose (0.2%) or pectin (1%).

The well-known coliphages T4, T7, P1 and lambda, and phage P22 *Salmonella typhimurium* were also used. The high titer lysates of these phages (except lambda) were obtained as the mentioned above on *Escherichia coli* B^E and C600, or *S. typhimurium* LT2, respectively. Phage lambda was isolated by the heat induction method [1] from the lysonenic *E. coli* strain M34(λcI_{857}S7).

The highly purified phage particles were obtained in the following way. The crude lysates without bacterial debris were concentrated during the one cycle of the high-speed centrifugation in the

rotor SW28 (28000 rev/min, 90 min, 10 °C). In the cases of stable phages the concentrated suspensions were additionally purified by equilibrium centrifugation in CsCl gradient (1.4 and 1.65 g/cm^3) which was formed in SW50Ti rotor (40000 rev/min, 4 h, 10 °C). Residual bacterial debris in highly concentrated unstable phage suspensions was removed by additional sedimentation at the speed 11000 rev/min for 15 min.

In general, STMG (200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 100 μg/ml gelatin) [14], STM (200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$) and TM (10 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$) buffers were used for gathering, preparation and storaging of bacteriophage high-titered suspensions.

The heat inactivation [8] of the phage particles was carried out in T buffer (100 mM Tris-HCl, pH 7.5) where gelatin or ficoll were added in concentrations within 100-1600 μg/ml and 2-6%, respectively, to identify their stabilizing effect. The experiment was performed at the constant temperature of 57 °C ± 0.05 °C. The concentration of phage particles in the inactivating suspension reached 1x10^9 PFU\ml in each experiment series both with P1 and ZF40.

Results and Discussions

In this study we have estimated structural stability of phage ZF40 compared with coliphage P1, which is a representative of the same phage A1-morphotype. P1 is one of the largest phages of *E. coli* [9], so a lower structural stability of its virion was expected as compared with phages of the same morphotype, but with a smaller virion size, such as ZF40.

The heat inactivation was applied to both these phages to compare their structural stability at the same conditions in T buffer. It was shown that the heat inactivation kinetics of ZF40 phage particles was quite different from that one for P1 (Fig. 1). In the case of ZF40 a decline in the phage viability occurred in two stages. Rapid inactivation (Fig.1, graph ZF40, segment I) was observed in the first 5 min. During this time, the phage viability decreased by 99.4%. The second stage (Fig.1, graph ZF40, segment II) was characterized by slow heat inactivation, when the particles that still remained viable, had completely lost their biological activity.

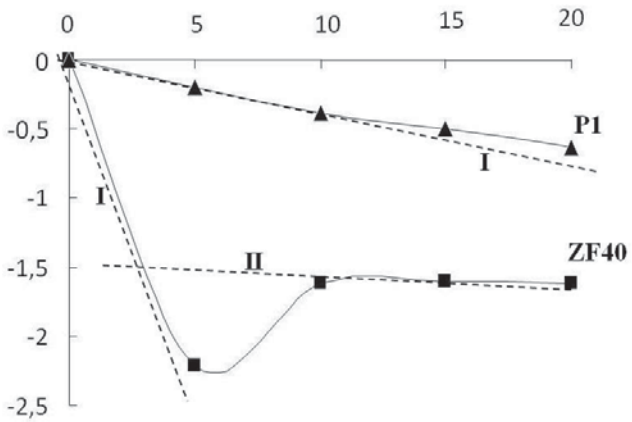

Fig.1. Heat inactivation of P1 and ZF40.

The figures I and II specify structural segments of the inactivation graphs. The presence of only one such segment suggested that the phage population consisted of virions having similar sensitivity to an inactivation agent. Such effect could be observed in the case of phage P1 heat inactivation. Another inactivation graph belonged to phage ZF40 and consisted of two different segments. Segment I, evidently, belonged to ZF40 virions with high heat sensitivity and fast heat inactivation (type I). The segment II evidently belonged to more stable and less heat sensitive ZF40 virions (type II).

Here and on the next figures, P/P_0 – phage survival index, where P – phage titer after heat inactivation, P_0 – initial phage titer

The inactivation graph of phage P1 (Fig.1) could be described by the first order reaction only and phage P1 particles were 10 times more resistant to heating than ZF40 ones. Moreover, the inactivation kinetics coefficient (Table), which was calculated for type II *ZF40* virions (for explan. see Fig. 1), coincided with that for phage P1 homogeneous population.

Table

Heat inactivation coefficients for phage P1 and phage ZF40

Bacteriophage	Virion type*	K***, $c^{-1}x10^{-3}$
P1	—**	2.7
ZF40	I	37
	II	2.5

* See explanation for Fig. 1

** P1 phage suspension used in the experiment was homogenous

*** The heat inactivation coefficient. The value of fidelity approximation (R^2) for the present data was from 0.94 till 1.0

One of the probable reasons of the phage ZF40 instability could be explained with an electron microscopy data shown on Fig.2. Evidently, the biological inactivation of ZF40 particles happened because of contraction of the phage tail (Fig.2, B). Probably this was due to the conformation changes of the basal plate, or because of collapsing of the phage head. The phage particles may also be destroyed by the osmotic shock that leads to swelling of the phage tails and sliding the tail sheaths [18]. On the Fig. 2, C, it was shown that ZF40 virions incurred more pervasive destruction after the tail contraction in comparison with phage T4. The destruction was manifested in increasing the numbers of empty phage heads and presence of fully destroyed capsids which appeared after conventional scheme of the high-titre phage preparation.

Thus, the presented data suggested that ZF40 virions are more labile in comparison with coliphage P1 and, as it was shown before, erwiniophages 49 and 59 [19]. Given the above mentioned, further research of phage ZF40 biology and its application as a molecular tool in Pectobacterium genetics were needed to develop a reliable system for the long-term storage and manipulations with the phage.

Long-term preservation of bacteriophages in collections, especially of stable phages with contracted tails (family Myoviridae) can be solved by the freezing of phage particles at -70 °C or by the liophilisation. Another way is the use of liquid or agar containing media on which bactreriophages propagated and storage of such preparations at +4 °C [1]. Traditionally, these media have in their composition protein substances, such as gelatin, to compensate energy changes in storage conditions. This method gives good results and partial decrease in a phage titre (approximately one order per year) can be initially limited through using of highly concentrated phage suspensions (10^{11}–10^{13} PFU\ ml). Most phages are stored directly in gradient forming solutions such as cesium chloride, sucrose, metrizamid and others without next dialysis. Conservation of temperate phages as prophages in natural or artificial lysogens is likely the most reliable approach for their long-term preservation.

Nevertheless, there are several significant problems in the long-term storage which were revealed during many years` experience with enterobacterial phages of different morphotypes. Firstly, the phage populations both *in vivo* and *in vitro* can be genetically unstable [3]. During the storage different mutations which are results of genomic rearrangement are accumulated in phage stocks. These mutations can lead to gathering of different mutants in phage populations which may be able to supply the wild type during following cultivations. For example, a sharp decrease in phage titer during storage can lead to phage stock enrichment by heat resistant mutants, and therefore, to the loss of the population authenticity. Secondly, there are the phages supersensitive to environment sharp fluctuations which may be destroyed by temperature, osmotic and electrostatic shock. Clearly, the genetic and physical instability of phages are closely linked together. Thirdly, separated components of phage virions (capsids, procapsids, tails, etc.) may also require long-term preservation in special storage conditions. Fourthly, some phage *nonsence*-mutants in structural proteins should be considered as a separate class of unstable phages.

Finally, our observations from storage process of different coliphages, phage P22 *S. typhimurium* and erwiniopahges 49, FE44 and ZF40 in T, TM, STM buffers and in LB medium allowed to divide them into three groups. Lambda and T4 high-titer phage stocks could be stored for years without any changes on condition that the lambda phage tail was stabilized by Mg^{2+} cations. It was important that the storage temperature was constant (+4 °C) and the phage lysates were kept in sterilized and hermetically sealed containers.

The second group included phages T7, P1, P22 and FE44, which concentration decreased 1-1.5 orders/year in any of the above mentioned systems. Among these viruses, phage FE44 was the most sensitive to temperature and osmotic shock.

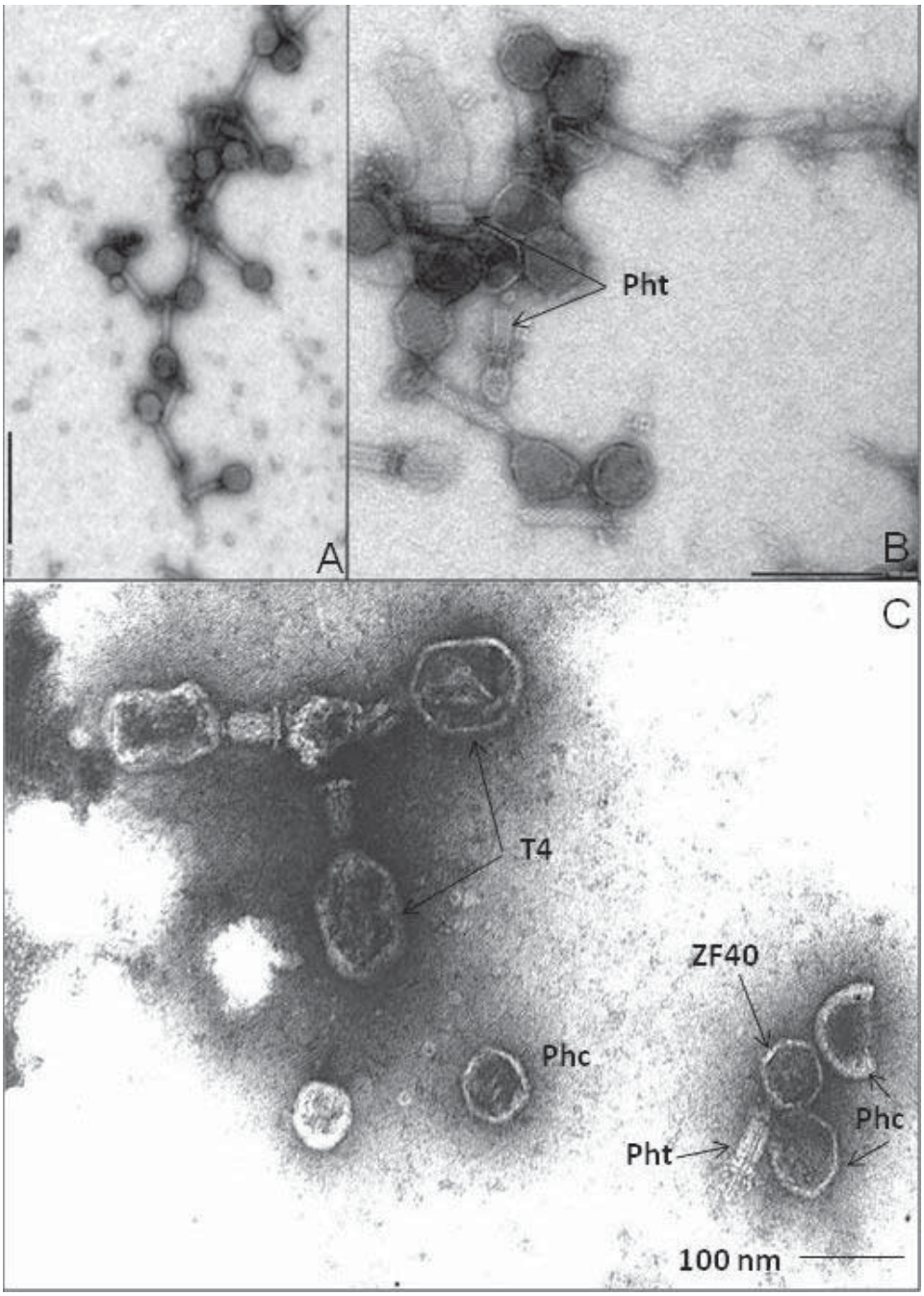

Fig. 2. Micrographs of erwiniophage ZF40 particles. A, B – ZF40 samples obtained after differential centrifugation; C – destroyed ZF40 particles. T4 – T4 virions with contracted tails; Phc – single capsids of phage ZF40; Pht – contracted tail of phage ZF40. Bar lines: A – 200 nm, B, C – 100 nm

Two other erwiniophages ZF40 and 49 could be included into the third group. These phages started to break down after the first month of storage in any of the buffer systems, and their concentration decreased 3-4 orders in high-titer stocks after one year of storage. Both these phages had specific sensitivity to osmotic shock and they rapidly decayed at all stages of concentration and purification procedures.

For developing a safe phage storage buffer system an experience of the phage T7 labile procapsids I and II study [14] was used. It allowed us to solve the problem of virion stability of the last

two phage groups. In that work the buffer STMG was proposed to stabilize the phage T7 procapsids. Leaving the same name, we have somewhat modified this buffer system, greatly increasing the concentration of magnesium cations to 10 mM $MgCl_2/MgSO_4$.

Using this modified STMG buffer at all stages of gathering, concentration and purification of unstable virions of ZF40, 49, T7, P1, R22 and FE44 phages we managed to avoid their uncontrolled destruction. Concentration and purification in CsCl gradient and subsequent dialysis of the phage samples which were dissolved in STMG, prevented T7 and P1 shocking, and decreased collapsing of ZF40, 49 and FE44 virions. When STMG buffer and gelatin (100-200 μg/ml) for LB were used the frequency of spontaneous *clear*-mutants of temperate bacteriophages 49 and 59 was significantly restricted. Additional research showed that the frequency for phage ZF40 was reduced approximately 1000 times.

The dependence of phage survival (P/P_0) on the concentration of gelatin in the buffer was studied to evaluate its stabilizing effect on ZF40 virions. It was found that during 30 min at 57 °C the phage survival in T-buffer decreased to 0.57% (Fig. 3). The gradual increase in gelatin concentration, ranging from 200 μg/ml, led to a linear growth of the survival. Maximal stabilization effect on the phage particles was observed at gelatin concentration of 800 μg/ml when the phage survival reached 4.6%, that exceeded eight times the original value of %(P/P_0). Additional studies demonstrated that the effective concentration of gelatin for the most efficient compensation of the temperature effect on the virions was 400-800 μg/ml, but the effectiveness depended on a number of factors, including the phage concentration. The behavior of the curve shown on Fig. 3 was rather difficult to analyze. It was especially difficult to explain a decrease of the stabilization effect to the initial level when the concentration of gelatin was gradually increasing to 1200 and 1600 mg/ml. Perhaps at these concentrations the gelatination took place, and the stabilizer was unable to shield the particles. Such undesirable effect was also observed when the medium with gelatin was exposed at lower temperatures from +4 to -2 ° C. It was observed that 0.01 - 0.1% LB and TM gelatin solutions were quite muddy independent of the presence of phage particles in them. At high concentrations of phage particles (10^{11} - 10^{12} PFU/ml) the stabilizer behavior may increase the virion aggregation and, partially reduce virion resistance.

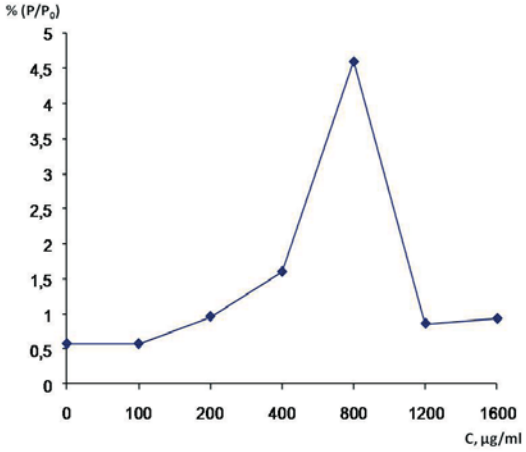

Fig. 3. Effect of gelatine concentration (C, μg/ml) in T buffer on phage ZF40 survival index (P/P_0) at 57 °C during 30 min

Notwithstanding the foregoing, the noted comment which concerned the gelatinized buffer solutions, including STMG buffer, in no way could reduce their advantages for long-term storage of bacteriophages. But, these buffers were of little use for long-term conservation of highly concentrated phage stocks.

Special research showed that the protein stabilizers of phage particles could be replaced by polysaccharide substances such as manitol, metryzamid, ficoll and others. It was found that after 1-2-year storage of ZF40 and FE44 phage particles in buffers containing 50-200 mM NaCl, 10-100 mM Tris-HCl, pH 7.5, and 10 mM $MgSO_4$ with 3 - 6% ficoll exhibited properties similar to STMG ones. As it can be seen on the Fig. 4, the stabilizing effect of ficoll was slowly increasing depending on its concentration. The study did not show any abnormal effects of the maintaining buffer system. We

have found that the native ZF40 DNA could be extracted from samples which had been stored in the STMF buffer during 1-2 years at +4 °C. Such samples also could serve as sources for obtaining phage mutants of *clear*-, *del*- or *ts*-types.

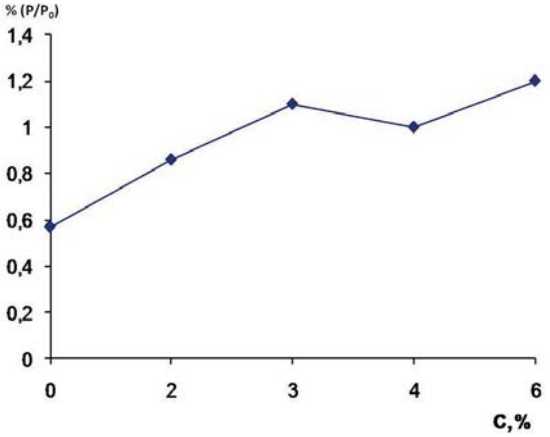

Fig. 4. Effect of ficoll concentration (C, %) in T buffer on phage ZF40 survival index (P/P_0) at 57 °C during 30 min

Thus the proposed STMF buffer could be successfully used for long-term preservation of unstable enterobacterial phages at temperatures from +4 to -2 °C. The storage conditions allowed to retain genetic and physical stability of phage population, and made it possible to use unstable phage virions and their individual components for molecular studies at any time.

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ДОВГОСТРОКОВЕ ЗБЕРІГАННЯ НЕСТАБІЛЬНИХ БАКТЕРІОФАГІВ ЕНТЕРОБАКТЕРІЙ

Р е з ю м е

Бактеріофаги є невід`ємними компонентами бактеріальних спільнот. Крім того, вони мають важливе практичне значення і використовуються в різних сферах. Тому зберігання фагових колекцій та фагоспецифічних бактеріальних штамів є актуальним завданням для біологів. Проблеми, які виникають при довгостроковому зберіганні фагів, все ще повністю не вирішені. Складності пов`язані, головним чином, зі структурною нестабільністю віріонів та прискореною генетичною мінливістю фагових геномів як *in vivo,* так і *in vitro*. В запропонованій роботі представлено результати десятирічних спостережень за структурною цілісністю нестабільних бактеріофагів. Запропоновано метод для їх довгострокового зберігання, який полягає в оптимізації буферної системи STMG (200 мМ NaCl, 10 мМ Tris-HCl, pH 7.4, 1 мМ $MgCl_2$, 100 мкг/мл желатину) [*Serwer P., Pichler M.E. Electrophoresis of bacteroiphage T7 capsids in agarose gels// J.Virol. – 1978. – V.28, N3. – P.917-928*] за рахунок збільшення концентрації желатину або його заміщення на фікол в концентрації 2 – 6 % та збільшення кількості іонів Mg^{2+} до 10 мМ. Запропонований склад буфера дозволив зберегти структурну цілісність нестабільних фагових віріонів під час їх довгострокового зберігання.

Ключові слова: бактеріофаги, довгострокове зберігання, структурна нестабільність, желатин, фікол.

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ДОЛГОСРОЧНОЕ ХРАНЕНИЕ НЕСТАБИЛЬНЫХ БАКТЕРИОФАГОВ ЭНТЕРОБАКТЕРИЙ

Р е з ю м е

Бактериофаги являются неотъемлемыми компонентами бактериальных сообществ. Они также имеют важное практическое значение и используются в различных областях человеческой деятельности. Поэтому хранение фаговых коллекций и фагоспецифических бактериальных штаммов является актуальным заданием для биологов. Проблемы, которые возникают при длительном хранении фагов, все еще полностью не решены. Сложности связаны, главным образом, со структурной нестабильностью вирионов и ускоренной генетической изменчивостью фаговых геномов как *in vivo,* так и *in vitro*. В предложенной работе представлены результаты десятилетних наблюдений за структурной целостностью нестабильных бактериофагов. Предложен метод для их длительного хранения, который заключается в оптимизации буферной системы STMG (200 мМ NaCl, 10 мМ Tris-HCl, pH 7.4, 1 мМ $MgCl_2$, 100 мкг/мл желатина) [*Serwer P., Pichler M.E. Electrophoresis of bacteroiphage T7 capsids in agarose gels// J.Virol. – 1978. – V.28, N3. – P.917-928*] за счет увеличения содержания желатина или его замены на фикол в концентрации 2 – 6 % и увеличения количества ионов Mg^{2+} до 10мМ. Предложенный состав буфера позволил сохранить структурную целостность нестабильных фаговых вирионов при их долгосрочном хранении.

К л ю ч е в ы е с л о в а : бактериофаги, долгосрочное хранение, структурная стабильность, желатин, фикол.

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