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CELL BIOPHYSICS

Determination of the Spectrum of Lytic Activity of Bacteriophages by the Method of Acoustic Analysis

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Abstract—The changes in the electroacoustic parameters of a cell suspension due to the interaction between cells and bacteriophages were studied both in pure culture and in the presence of extraneous microflora. It was shown that the specific changes in the electroacoustic parameters of a cell suspension under the influence of bacteriophage occur only in the microbial cells that are sensitive to this bacteriophage. A sensor unit allowed us to distinguish the situation where bacterial cells are infected with specific bacteriophages of the control experiments from the situation without such infection. An approximate criterion for specific interactions between bacteriophages and cells in the suspension was developed. According to this criterion, the change in electrical impedance of a sensor unit must not be less than ~1%. The standard microbiological technique of sowing the cells infected with bacteriophages on solid nutrient media was used as a control. The possibility of using the method of electroacoustic analysis to determine the spectrum of lytic activity of bacteriophages has been shown for the first time. The results that we obtained can be used to develop a new express method for determining the sensitivity of microbial cells to bacteriophages.

Keywords: spectrum of lytic activity, bacteriophages, microbial cells, electroacoustic method of analysis

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Bacteriophages can be a biological receptor for the detection of microbial cells [1] and are interesting for the study of the interaction between bacteriophages and microbial cells. In addition, bacteriophages can be used for detection of many pathogens of various infectious diseases of humans, animals, and plants [2] and are an effective tool for controlling these pathogens (including those that are resistant to antibiotics) [3–6]. The efficiency is determined by the specific effects of bacteriophages, as well as by the simplicity of making the preparations and their low cost compared to the development of novel antibiotics against resistant bacterial strains. It is noteworthy that thousands of people were cured of dysentery, typhus, cholera, and other infectious diseases in the 1920s using bacteriophage preparations. However, the application of bacteriophages as therapeutic and preventive drugs against infectious diseases was stopped in the 1940s, after the discovery of antibiotics.

The interest in bacteriophages has arisen again due to the increasing number of antibiotic-resistant microorganisms. Nevertheless, the basic condition of successful application of bacteriophages is pathogen sensitivity to the respective bacteriophage, i.e., phage specificity. There are several methods for determining the sensitivity of bacteria to bacteriophages, including:

—the standard microbiological methods [7, 8];

—the method of electro-orientation spectroscopy based on recording the changes in the optical properties of a microbial suspension under the influence of a variable electric field;

—fluorescence spectroscopy based on the introduction of a membranotropic probe into the system and recording the fluorescence intensity [9]; and

—bacteriophage-based biosensor techniques [10, 11].

Recently, the investigation of the electrophysical properties of cells has been actively developed due to some advantages of the electrophysical methods of analysis. First, there is a close relationship between the electrophysical characteristics of cells and processes of their vital activity; second, these characteristics can be quickly measured without destroying the cells and cell structures, allowing the study of cell processes in situ [12, 13]. Acoustic methods of analysis are attracting increasing attention of researchers for the analysis of biological interactions, since they are characterized by high sensitivity and a minimal time of analysis. The piezoelectric resonators with a transverse (lateral) electric field are more sensitive to the contacting liquid, in contrast to the common resonators with a longitudinal (normal) field, because they respond to the changes both in its viscosity and in its conductivity. Many articles and patents are devoted to these resonators and their application for the solution of biotechnological problems [14–17].

Since the assay of the lytic activity of bacteriophages is a rather long procedure, it would be extremely important to develop a method for the express analysis of microbial sensitivity to bacteriophages. Hence, this work was aimed at developing the method of electroacoustic analysis of cell suspensions for the determination of the spectrum of bacteriophage lytic activity and the solution of the problem of phage sensitivity of microbial cells.

MATERIALS AND METHODS

Microorganisms. The following microorganisms were used in the work: *Escherichia coli* strains XL-1, K-12, B-878, BL-Ril, pHEN1, pUC18, and pBR325, *Azospirillum brasilense* Sp7 (IBPPM 150), which were obtained from the collection of rhizosphere microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms of the Russian Academy of Sciences. Other strains were obtained from the collection of the laboratory of the Saratov Research Institute of Biocatalysis: *Pseudomonas putida* BA-11 (VKPM B-6707), *Pseudomonas putida* C-11 (VKPM B-6708), and *Acinetobacter calcoaceticum* A-122.

The microorganisms were stored at 4°C on Petri dishes with a solid nutrient LB medium containing 3% agar–agar (for the cells of *Escherichia coli*). Microbial cells were subcultured every 2 weeks.

The cultivation of microorganisms. The bacteria were cultivated in liquid LB medium [18] containing (g/L): NaCl (Becton, Dickinson & Co., United States), 10.0; peptone (Becton, Dickinson & Co., United States), 5.0; yeast extract (DIFCO, United States), 5.0. The semiliquid LB medium contained 0.7% agar–agar; the solid medium contained 1.5 and 3% agar–agar.

The bacterial cultures were grown in 250-mL Erlenmeyer flasks in liquid LB medium. The cells were incubated on a rotary shaker at an agitation rate of 160 rpm at 30 ± 1°C for 18–20 h.

The infection of cells with bacteriophage. The cells of *Escherichia coli* XL-1 were infected with the M13K07 filamentous phage of the *Inoviridae* family. A commercial preparation of M13K07 (Stratagene, Sweden) that is resistant to kanamycin was constructed on the basis of the wild-type phage M13 [18, 19]. To infect the *E. coli* XL-1 culture with the bacteriophage, it was inoculated from a separate colony on a dish with agarized LB medium containing 12.5 μg/mL of tetracycline into 2 mL of LB medium and incubated overnight under continuous aeration at 37°C; then one-tenth of the overnight culture was reinoculated into a fresh medium of the same composition and grown to the exponential growth phase at 37°C under aeration [18, 19]. On reaching the early logarithmic growth phase (OD_{600} = 0.5–0.6, corresponding to $7 \cdot 10^8$ cells/mL), the aeration was stopped for 30–40 min for the regeneration of F-pili and the suspension was incubated in a thermostat at 37°C. The concentration of microorganisms was calculated by the standard method using light microscopy. The microbial cells were infected by introducing the bacteriophage at 20 phage particles per one cell. On addition of the phages, the culture was incubated at 37°C in a thermostat without shaking for the sorption of phage particles on the surface of pili.

The isolation and characterization of bacteriophages. The bacteriophages were exposed to low temperature for isolation [18]. The thus-grown culture of microorganisms was cooled in a refrigerator at 4°C for 1.5–2 h to stimulate the release of bacteriophages from the cells. It was then centrifuged at 2500 *g* for 40 min. One-fifth of a 20% polyethyleneglycol solution (PEG6000, Pancreac, Spain) with 1.6 M NaCl (PEG/NaCl) was added to the supernatant. The flask with the supernatant was then covered with ice and put into a refrigerator at 4°C for 2 h. After the specified time period, the centrifugation was performed at 12000 *g* for 30 min, the supernatant was poured out, and the test tube was turned upside down, placed on filter paper, and dried for approximately 30 min. Next, 1 mL of the TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5–8.0) was added to the precipitate, resuspended, and centrifuged at 10000 *g* for 5 min. The supernatant was placed into a sterile vessel, with the addition of one-fifth of the volume of PEG/NaCl, and the formed precipitate was quickly stirred and centrifuged at 6500 *g* for 5 min. The resultant precipitate was dissolved in 1 mL of the TE buffer. The test tubes with phage suspension were stored in a freezing chamber at –20°C.

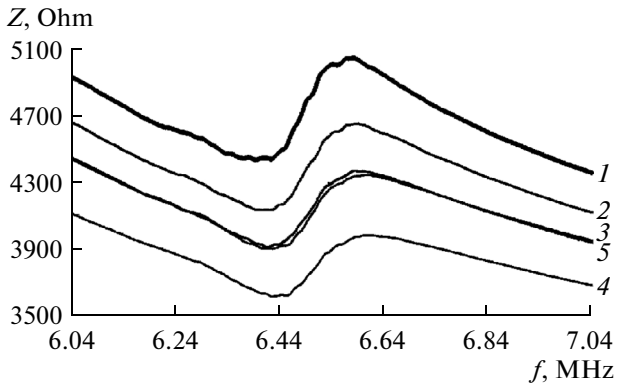

Fig. 1. The frequency dependencies of the modulus of electrical impedance of resonator after the infection of *E. coli* XL-1 cells by different levels of the M13K07 bacteriophage: the cell suspension without the bacteriophage (*1*); the cell suspension with the bacteriophages: 5 phages per 1 bacterium (*2*); 10 phages per 1 bacterium (*3*); 20 phages per 1 bacterium (*4*); 30 phages per 1 bacterium (*5*).

Determination of the concentration of phage particles. The quantity of phage particles was determined by spectrophotometry with a Specord BS-250 (Analytik Jena, Germany) in a 1-mL cuvette. Based on the fact that the value of 30 optical units corresponds to the value of $2 \cdot 10^{14}$ phage particles/mL [19], the calculations could be made by the formula: $(A_{269} - A_{320}) \cdot 5 \cdot 10^{14}/15$, where A_{269} is the optical density of the suspension under electromagnetic radiation with a wavelength of 269 nm and A_{320} is the optical density of the suspension at a wavelength of 320 nm.

Electroacoustic-sensor analysis. All experiments on the changes in the mechanical and electrical properties of microbial cell suspensions during biospecific interaction with phages were performed with a specially-manufactured sensor that is based on a piezoelectric resonator with a lateral electric field in the frequency range of 6–7 MHz. This resonator was made of a 0.5 mm-thick X-cut lithium niobate plate. Two rectangular electrodes ($5 \cdot 10$ mm^2) with a 3-mm gap between them were applied to the lower side of the plate. The area around the electrodes and some part of the electrodes were coated with a special varnish that damps parasitic Lamb waves [20] and provides a sufficiently high quality factor (~630). A liquid cell (~1-mL) was stuck to the upper side of the plate.

Ready bacterial cell suspension without bacteriophage was put into the above liquid cell for analysis; the modulus of the electrical impedance of the sensor was measured with an Agilent 4285A precision LCR meter. A certain number of bacteriophages was then added into the suspension and the measurements were repeated. The electrical impedance modulus of the sensor was used as the analytical signal because this parameter is most sensitive to the changes in both conductivity and viscosity of the analyzed liquid [21].

RESULTS AND DISCUSSION

The possibility of the electroacoustic detection of biological interactions was assessed with the following microorganisms that were used as model systems: *E. coli* strains XL-1, K-12, B-878, BL-Ril, pHEN1, pUC18, pBR325, and the bacteriophage M13K07, which exerts a specific effect on the male (F+) cells of *E. coli*.

Bacteriophage M13 is an *E. coli*-specific filamentous phage that infects the male cells of *E. coli* without their lysis [22, 23]. Since the cells of this strain bear an F-episome and produce F-pili, they can be infected with the M13K07 phage. The infection of *E. coli* cells that bear F-pili with the M13 phage is a well-studied process [24, 25]. The infection starts with the interaction of the bacteriophage minor coat protein g3p with bacterial F-pili, which are the primary phage receptors, and then with the integral membrane protein TolA [26]. The phage interaction leads to substantial changes in microbial cells that is determined by penetration of viral DNA into the cytoplasm of *E. coli* cells, while the coat protein (g8p) is incorporated into the inner cytoplasmic membrane. It may be supposed that each stage of phage infection of microbial cells will change the electroacoustic parameters of cell suspensions.

The frequency range of 6–7 MHz was chosen in preliminary experiments on the optimization of analytical conditions (the choice of the measuring frequency, the time of interaction, and the number of microbial cells in the measuring cell); the duration of the experiment was ~10 min. Microbial cells were introduced into the measuring cell at a quantity of 10^4 cells/mL.

An electroacoustic sensor was used to study the cell suspension of *E. coli* strain XL-1 that was infected with the M13K07 bacteriophage. Since transfection occurs via the F-pili on the bacterial surface, which can be formed at no less than 37°C, the cell culture was grown at this temperature and the suspension was infected with the phage at 37°C for 10 min, with the addition of different quantities of M13K07 phage particles (5, 10, 20, and 30 phages per 1 bacterial cell). The time of analysis was chosen in accordance with the previous data. These studies showed that the sensor distinguishes between the experiments where bacterial cells are infected with specific bacteriophages and the control without such infection. Figure 1 shows the dependences of the electrical impedance modulus of the sensor, when the liquid cell contained the suspension of *E. coli* XL-1 cells at 10^4 cells/mL, with the addition of different numbers of bacteriophages. The data show that the electrical impedance changes when the level of bacteriophage in a sample is five viral particles per cell. With an increase in the specific quantity of the bacteriophage that is added to a cell suspension, the impedance modulus decreases over the entire range until the level of the bacteriophage reaches

20 phages/cell and then increases again with a further increase in the quantity of added phage particles. From a physical point of view, this can be explained as follows. On the addition of bacteriophage, the electrical conductivity of the suspension first increases, which results in a monotonous decrease of the modulus of the electrical impedance of the piezoelectric resonator right up to the concentration of 20 phages/cell. A further increase in the quantity of bacteriophages leads to higher viscosity and the respective enhancement of electrical impedance. As a result of these two differently directed processes [22], the curves that correspond to the concentrations of 10 and 30 phages/cell actually coincide. The results show significant changes in electrical impedance in the presence of the bacteriophage M13K07 in the *E. coli* XL-1 cell suspension, which suggests a specific interaction between the bacteriophage and the XL-1 culture. Since the electroacoustic parameters of the cell suspension demonstrate the maximum changes after the addition of bacteriophages at the level of 20 phages per cell, the same conditions were used in further experiments.

At the next stage of research, it was necessary to test the bacteriophage selectivity against the cells of other microorganisms (*E. coli* K-12). The choice of the *E. coli* K-12 was made due to the fact that it is a parent strain for *E. coli* XL-1 [27]; it is very likely that the M13K07 phage can also infect the cells of the K-12 strain. The results that are presented in Fig. 2a confirm this assumption. The difference in the values of the electrical impedance modulus at the maximum frequency was ~1%.

Thus, the recorded changes in electroacoustic parameters of cell suspension after the addition of bacteriophage lead to the conclusion that M13K07 infects the cells of *E. coli* strains XL-1 and K-12, i.e., these microorganisms are sensitive to this bacteriophage.

At the next stage, it was necessary to eliminate the possibility of nonspecific interaction between the M13K07 bacteriophage and the cells of other strains. The cells of *E. coli* strains B-878, BL-Ril, pMMB33, pHEN1, pUC18, and pBR325 were used as control suspensions. The analytical conditions were the same as in the experiment with XL-1 cells. Figure 2b shows that the frequency dependence of the modulus of impedance for *E. coli* B-878 cells actually did not change after the addition of the M13K07 bacteriophage, i.e., the cells of *E. coli* strain B-878 are resistant to the M13K07 bacteriophage. Similar results were obtained for the cells of *E. coli* strains BL-Ril (Fig. 2c) and pHEN1, pUC18, pBR325.

Thus, it has been shown that the changes in the electroacoustic parameters of cell suspensions that were infected with bacteriophage are considerably different for microorganisms that are sensitive and insensitive to this bacteriophage.

One of the most important features of the development of a new technique for determining the spectrum of phage lytic activity is testing it in several objects. Therefore, we investigated the possibilities of the method of electroacoustic analysis of microbial suspensions for assessing the M13K07 specificity to the cells of *Pseudomonas putida* strains C-11 and BA-11 and *Acinetobacter calcoaceticum* A-122. The experimental conditions were similar to those for *Escherichia coli* cells. It was shown that the modulus of electrical impedance did not change after the infection of the cells with the M13K07 bacteriophage; consequently, these cells are resistant to the bacteriophage under study.

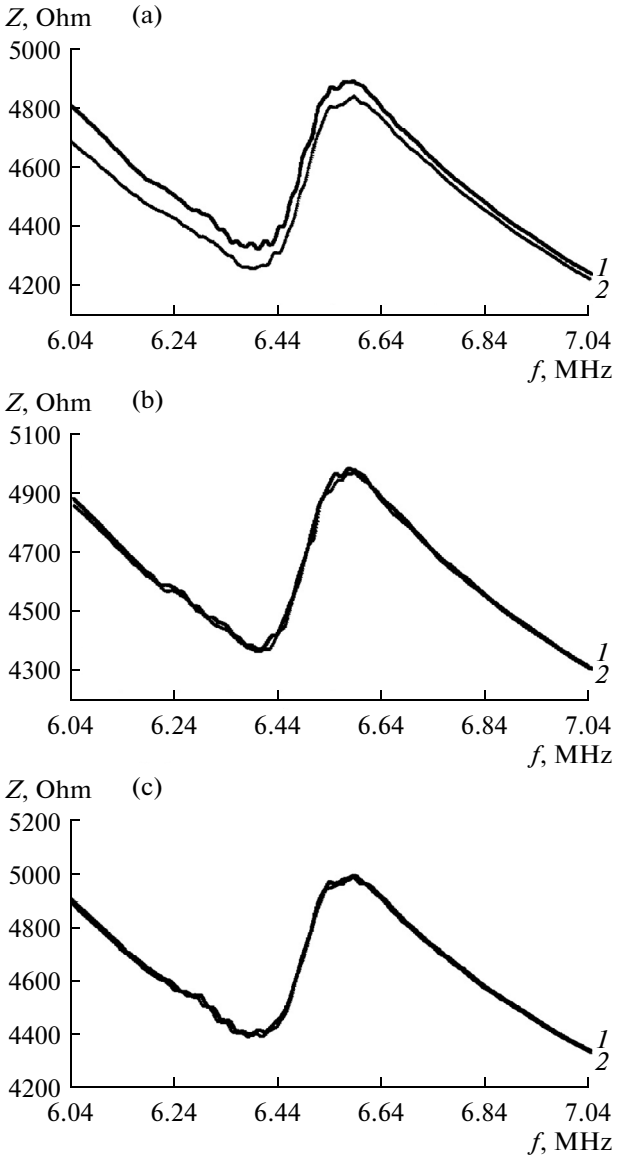

Fig. 2. The frequency dependencies of the modulus of electrical impedance of resonator after the infection of *E. coli* K-12 (a), *E. coli* B-878 (b), and *E. coli* BL-Ril (c) cells by the M13K07 bacteriophage (20 phages per 1 bacterium): uninfected cells (*1*); infected cells (*2*).

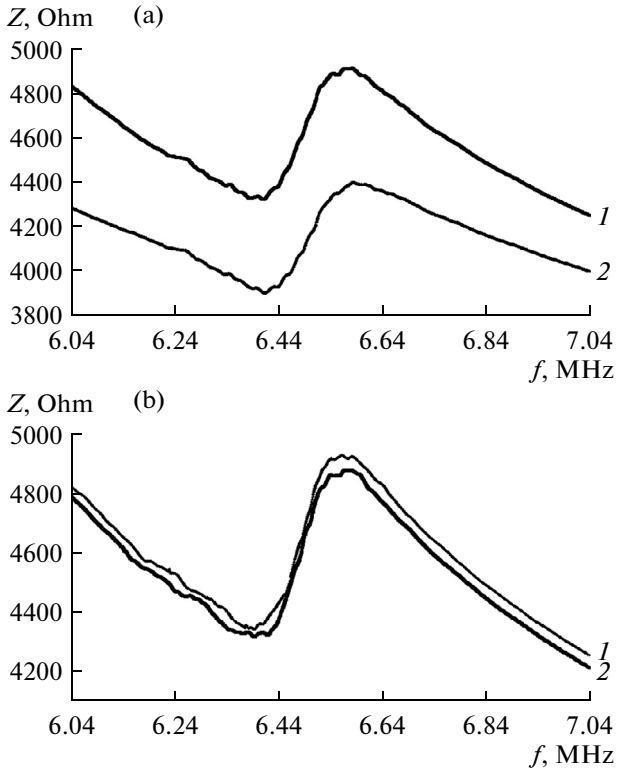

Fig. 3. The frequency dependencies of the modulus of electrical impedance of resonator for the mixed cell suspension of *E. coli* B-878, *E. coli* BL-Ril, *A. brasilense* Sp7, and *E. coli* XL-1 (a) and for the cell suspension of *A. brasilense* Sp7 (b) after the infection by the M13K07 bacteriophage (20 phages per 1 bacterium): uninfected cells (*1*); infected cells (*2*).

Further, it was interesting to compare the data that were obtained by the method of electroacoustic analysis of microbial suspensions with the results of the standard microbiological technique. The process of bacteriophage transfection into bacterial cells was controlled by inoculating phage-infected cells onto the LB medium with kanamycin, since the M13K07 phage carried kanamycin-resistance factors [19]. Good growth of XL-1 and K-12 cells was observed on the Petri dish with the kanamycin-containing medium, which is the evidence of phage infection of the cells and their sensitivity to the M13K07 bacteriophage. However, the B-878, BL-Ril, pHEN1, pUC18, and pBR325 strains, as well as *P. putida* strains C-11 and BA-11, and *A. calcoaceticum* A-122, showed no cell growth on the kanamycin-containing medium, i.e., the cells of these strains are resistant to the M13K07 phage.

In practice, it is often necessary to obtain the information about the bacteriophage sensitivity of the studied bacterial strain in the presence of extraneous microflora. Therefore, we investigated the possibility of obtaining analytical signals in the presence of extraneous microflora using an electroacoustic sensor. To this end, the M13K07 bacteriophage was added to a cell suspension of *E. coli* strains XL-1, B-878, BL-Ril, and *A. brasilense* Sp7 mixed at a ratio of 1 : 1 : 1 : 1; the analytical conditions were similar to those with the mono cell culture. *A. brasilense* Sp7 was chosen as a ballast culture due to its different taxonomic position and a cell size that is similar to that of *E. coli*. A mixed cell suspension of *E. coli* XL-1, B-878, BL-Ril, and *A. brasilense* Sp7 without the bacteriophage was used as a control. Figure 3a shows a significant change in the analytical signal on the addition of the M13K07 bacteriophage to the mixed cell suspension. The results of the infection of *A. brasilense* Sp7 cells with the M13K07 bacteriophage are presented in Fig. 3b.

Here it should be noted that the minor difference between the frequency dependencies for the suspensions with and without bacteriophages for the control experiments that are shown in Fig. 3b is not related to the phage infection of bacterial cells, although the maximum change in the modulus of elasticity in the vicinity of resonance is ~1%, as in the case shown in Fig. 2a. Based on this result, the criterion for specific interaction can be approximately defined as a no less than ~1% change in the modulus of the electrical impedance of the resonator with the addition of a certain quantity of bacteriophages to a cell suspension. However, one can see that the dependencies in Fig. 2a are more divergent in other points of the range compared to the curves in Fig. 3b. Hereafter, not only the resonance frequency but also the frequency set in a definite range close to the resonance frequency should be taken into consideration in order to determine this criterion.

Thus, our studies have shown that the sensor distinguishes between the situations where bacterial cells are infected with specific bacteriophages, and the control experiments without such infection. The changes in the electroacoustic parameters of a cell suspension during its interaction with bacteriophages have been studied both in a pure culture and in a mixed cell suspension. An approximate criterion for a specific interaction between bacteriophages and cells in the analyzed suspension has been developed: the change in the modulus of electrical impedance of the sensor should not be less than ~1% after the addition of a certain number of bacteriophage to cell suspension. The results can be used to create an express method for determining the spectrum of phage lytic activity and the phage sensitivity of microbial cells.

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REFERENCES

1. H. Zhu, I. M. White, and J. D. X. Suter, Biosens. Bioelectron. **24**, 461 (2008).

2. T. K. Lu, J. Bowers, and M. S. Koeris, Trends Biotechnol. **31**, 325 (2013).
3. A. V. Letarov, A. K. Golomidova, and K. K. Tarasyan, Acta Naturae **2**, 66 (2010).
4. N. V. Pimenov, V. V. Subbotin, and N. V. Danilevskaya, Ross. Vet. Zh. **6**, 6 (2013).
5. J. B. Jones, L. E. Jackson, B. Balogh, et al., Annu. Rev. Phytopathol. **45**, 245 (2007).
6. X. Wittebole, S. De Roock, and S. M. Opal, Virulence **5**, 209 (2014).
7. M. H. Adams, *Bacteriophages* (Interscience Publ., New York, 1959; Medgiz, Moscow, 1961).
8. J. S. Kumar and B. Dhar, Biol. Fertil. Soils **46**, 681 (2010).
9. E. L. Zhilenkov, I. G. Shemyakin, V. M. Fomchenkov, et al., Microbiology (Moscow) **67**, 550 (1998).
10. S. Balasubramanian, I. Sorokulova, V. Vodyanoy, and A. Simonian, Biosens. Bioelectron. **22**, 948 (2007).
11. S. Li, Y. Lib, H. Chenb, S. Horikawaa, et al., Biosens. Bioelectron. 26, 1313 (2010).
12. A. I. Miroshnikov, V. M. Fomchenkov, and A. Yu. Ivanov, *Electrophysiological Analysis and Cell Sorting* (Nauka, Moscow, 1986) [in Russian].
13. J. Gimsa and D. Wachner, Biophys. J. **75**, 1107 (1998).
14. B. D. Zaitsev, I. E. Kuznetsova, A. M. Shikhabudinov, et al., IEEE Trans. Ultrason., Ferroelectr. Freq. Control **59**, 963 (2012).
15. O. I. Guliy, B. D. Zaitsev, I. E. Kuznetsova, et al., Biophysics (Moscow) **57**, 336 (2012).
16. R. C. Smythe and H. F. Tiersten, IEEE Trans. Ultrason. Ferroelectr. Freq. Control **35**, 435 (1988).
17. O. I. Guliy, B. D. Zaitsev, I. E. Kuznetsova, et al., Microbiology (Moscow) **82**, 215 (2013).
18. T. Maniatis, E. F. Frich, and J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Cold Spring Harbor, NY; Mir, Moscow, 1984).
19. G. P. Smith and J. K. Scott, Methods Enzymol. **217**, 228 (1993).
20. B. D. Zaitsev, I. E. Kuznetsova, A. M. Shikhabudinov, and A.A. Vasil'ev, Pis'ma Zh. Techn. Fiz., **37**, 27 (2011).
21. B. D. Zaitsev, A. M. Shikhabudinov, A. A. Teplykh, I. E. Kuznetsova, Uch. Zap. Fiz. Fakul't. MGU, 2014, 5, 145330.
22. D. A. Marvin, R. D. Hale, C. Nave, and M. Helmer-Citterich, J. Mol. Biol. **235**, 260 (1994).
23. S. A. Overman, M. Tsuboi, and G. J. Thomas, J. Mol. Biol. **259**, 331 (1996).
24. E. M. Click and R. E. Webster, J. Bacteriol. **179**, 6464 (1997).
25. L. W. Deng, P. Malik, and R. N. Perham, Virology **253** (2), 271 (1999).
26. H. Endemann, J. Mol. Biol. **250**, 496 (1995).
27. P. Kuhnert, J. Nicolet, and J. Frey, Appl. Environ. Microbiol. **61**, 4135 (1995).

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