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Determination of *Azospirillum brasilense* Cells with Bacteriophages by Electrooptical Analysis of Microbial Suspensions

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Abstract—The dependence of changes in the electrooptical properties of *Azospirillum brasilense* cell suspension Sp7 during interaction with bacteriophage ΦAb-Sp7 on the number and time of interactions was studied. Incubation of cells with bacteriophage significantly changed the electrooptical signal within one minute. The selective effect of bacteriophage ΦAb on 18 strains of bacteria of the genus *Azospirillum* was studied: *A. amazonense* Am1i4, *A. brasilense* Sp7, Cd, Spl07, Sp245, Jm6B2, Brl4, KR77, S17, S27, SR55, SR75, *A. halopraeferans* Au4, *A. irakense* KBC1, KA3, *A. lipoferum* Sp59b, SR65 and RG20a. We determined the limit of reliable determination of microbial cells infected with bacteriophage: ~10^4 cells/mL. The presence of foreign cell cultures of *E. coli* B-878 and *E. coli* XL-1 did not complicate the detection of *A brasilense* Sp7 cells with the use of bacteriophage ΦAb-Sp7. The results demonstrated that bacteriophage ΦAb-Sp7 can be used for the detection of *Azospirillum* microbial cells by the electrooptical analysis of cell suspensions.

Keywords: *Azospirillum brasilense*, bacteriophages, electrooptical analysis
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INTRODUCTION

Gram-negative, growth-stimulating bacteria of the genus *Azospirillum* play an important role in nitrogen fixation. *Azospirillum brasilense* attracts the attention of researchers as a model object for the study of associative and endophytic rhizosymbioses formed by bacteria and higher plants [1–5]. The composition and abundance of associative microbial flora depend on seasonal fluctuations and anthropogenic impacts. In this regard, the development of methods, including detection methods for *Azospirillum* cells, which allow monitoring of the rhizosphere bacteria, is one of the tasks of modern environmental microbiology [6].

Viruses of the bacteria infecting a microbial cell can be used for the detection of these microorganisms. The recognition and attachment of bacteriophages to bacterial cells is performed via receptors on the cell surface. This is the basis for the majority of microbial cell detection methods that involve bacteriophages [7–8], including the widely used biosensor methods [9–12]. The detection of microbial cells via electrophysical analysis methods has been actively developed [11, 13, 14]. Thus, methods the electrooptical analysis of microbial suspensions are based on the detection of changes in the optical properties of a microbial suspension under an alternating electric field. The study is based on the measurement of the surface electrical properties of cells and cellular structures induced by an electric field. The interaction of charges upon the interface with an electric field changes the orientation of bacteria in the environment, which leads to changes in the optical properties of the suspension [15]. It was previously demonstrated that the interaction of antibodies and viruses with bacterial cells changes the electrooptical (**EO**) parameters of bacterial suspensions [16–18].

The goal of this study was the isolation of bacteriophage from microbial cells of *A. brasilense* strain Sp7 and the use of a bacteriophage for the detection of *Azospirillum* cells by the electrooptical analysis of cell suspensions.

MATERIALS AND METHODS

Bacterial Strains and Conditions for Bacterial Growth

We used *A. brasilense* strains Sp7, Cd, Spl07, Sp245, Jm6B2, Brl4, KR77, S17, S27, SR55, SR75, *A. amazonense* Aml4, *A. halopraeferans* Au4, *A. irakense* KBC1, KA3, *A. lipoferum* Sp59b, RG20a, and SR65; *Escherichia coli* strains B-878 and XL-1 obtained from the culture collection of the Institute of

Biochemistry and Physiology of Plants and Microorganisms. The microorganisms were stored at +4°C in Petri dishes with potato agar (for *Azospirillum* cells) on Petri plates with a solid LB culture medium containing 3% agar-agar (for *E. coli* cells). The cultures were subcultured every 14 days.

A liquid LB medium of the following composition (g/L) was used to culture the bacteria [19]: NaCl – 10.0 and peptone – 5.0 (Becton, Dickinson, and Company, France); and yeast extract (DIFCO, USA) – 5.0.

A medium of the following composition (g/L) was used to store *Azospirillum* (g/L): potato tubers – 200, agar – 30.

Bacterial cultures were grown in 250 mL Erlenmeyer flasks on LB liquid medium on a rotary shaker with vigorous agitation (160 rev/min) for 18–20 h at 30 ± 1°C.

Isolation and Characterization of Bacteriophages

Bacteriophage isolation was carried out by the method described in [19]. To stimulate the release of the bacteriophage from microbial cells, the culture was cooled to +4°C for 1.5–2 h, and the cells were then collected by centrifugation at 2500 *g* for 40 min. Polyethylene glycol (1/5 volume of 20% solution, PEG6000, Rapgeas, Spain) was added to the supernatant containing 1.6 M NaCl (**PEGNaCl**) and cooled to 4°C for 2 h. The precipitate was separated by centrifugation at 12000 *g* for 30 min and dried for 30 min. The pellet was suspended in 1 mL of 10 mM Tris-HC1 buffer, pH 7.5–8.0, containing 1 mM EDTA (**TE buffer**), and the insoluble portion was removed by centrifugation at 10000 *g* for 5 min. PEGNaC1 (0.2 mL) was added to the supernatant under sterile conditions, and the precipitate that formed was separated by centrifugation at 6500 *g* for 5 min and dissolved in 1 mL of TE buffer. The bacteriophage suspension was stored at –4°C.

Determination of the Number of Phage Particles

The number of phage particles was determined spectrophotometrically with a Specord BS-250 (Analytic Jena, Germany) in a 1 mm cuvette.

Preparation of Cells for Electrooptical Analysis

For electrooptical analysis, cells were washed three times with distilled water, centrifuged at 2800 *g* for 5 min, and then resuspended in a small volume of water (conductivity 1.6 μS/cm) and centrifuged at 110 *g* for 1 min to remove conglomerates. The resulting bacterial cell suspension in the supernatant was diluted with distilled water with an absorbance value of 0.4 at 670 nm, corresponding to a concentration of 4.5×10^8 cells/mL.

Electrooptical Analysis of Cell Suspensions

Measurement of the orientation spectra (**OS**) of cells was performed using an ELUS electrooptical analyzer (State Research Center for Applied Microbiology, Russia) with a discrete set of frequencies of the orienting electric field (740, 1000, 1450, 2000, and 2800 kHz) [20] at 670 nm; the intensity of electric field was 17 V/cm, and the application time was 16 s. The optical path length of the electrooptical cell was 8 mm, the volume was 1 ml, and the concentration of cells was 4.5×10^8 cells/mL. The number of cells was determined by the standard method on an "Ergaval" light microscope (Carl Zeiss, Germany).

The orientation spectrum was presented as the dependence of the optical density of the suspensions (δOD) at 670 nm on the frequency of the electric field in the propagation of unpolarized light along and across the orienting field. This difference was normalized to the optical density at 670 nm for randomly oriented cells. The measurements were made using relative units, which corresponded up to a constant of about $1–5 \times 10^{-32}$ to the anisotropy of the polarizability of particles with the dimension F/m^2.

All assays were performed at least in five replicates, and the results are presented as the mean values obtained with a standard deviation. The percentage error of measurements of standard samples was ±3%.

RESULTS AND DISCUSSION

Information about bacteriophages from bacteria of the genus *Azospirillum* is limited. The first studies on the isolation of bacteriophages from *Azospirillum* cells were published in the 1980s [21–23]. The isolation and investigation of 24 strains of bacteriophages of four bacterial species belonging to the genus *Azospirillum* [24], in particular, the bacteriophage of *A. brasilense* Sp7 cells, was described later. The microbial cells of this strain were used in this study to isolate bacteriophage ΦAb-Sp7.

Optimization of the measurement conditions of the EO parameters of a *A. brasilense* cell suspension of Sp7 included the selection of conditions for the application time of an electric field, voltage, and frequency. An electric field intensity equal to 17 V/cm was chosen, the time of application was 16 s, and the frequencies were 740, 1000, 1450, 2000, and 2800 kHz.

The study of the dynamics of changes of the magnitude of the EO signal of *A. brasilense* Sp7 cell suspension infected with ΦAb-Sp7 bacteriophage (20 bacteriophages per bacteria) showed a change in the EO signal. In further experiments, we used the same conditions.

The bacteriophage was added to the cell suspension *A. brasilense* Sp7 (10^8 cells/mL) and incubated for 1, 5, 10, 20, and 30 min at 37°C. The electrical conductivity of water in all cases was 1.6 μS/m. The EO signal changed after one minute when *A. brasilense* Sp7 cells

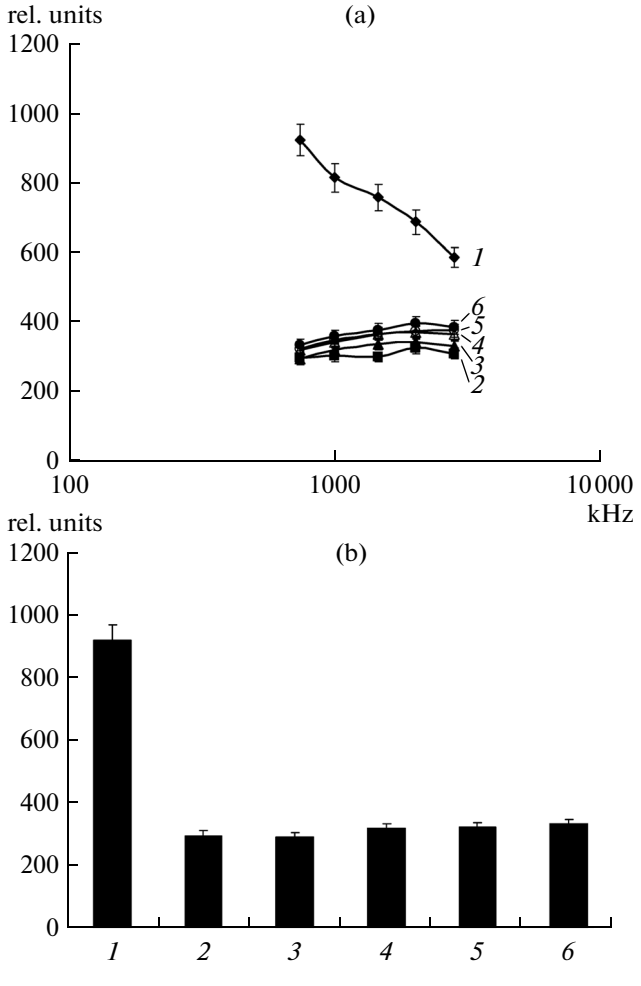

Fig. 1. Dynamics of changes in the EO signal of an *A. brasilense* Sp7 cell suspension infected with bacteriophage ΦAb-Sp7 at different frequencies of orienting field (a) and 740 kHz (b). (*1*) Uninfected cells (control), infecting time (min): 1 (*2*), 5 (*3*), 10 (*4*), 20 (*5*), 30 (*6*) minutes.

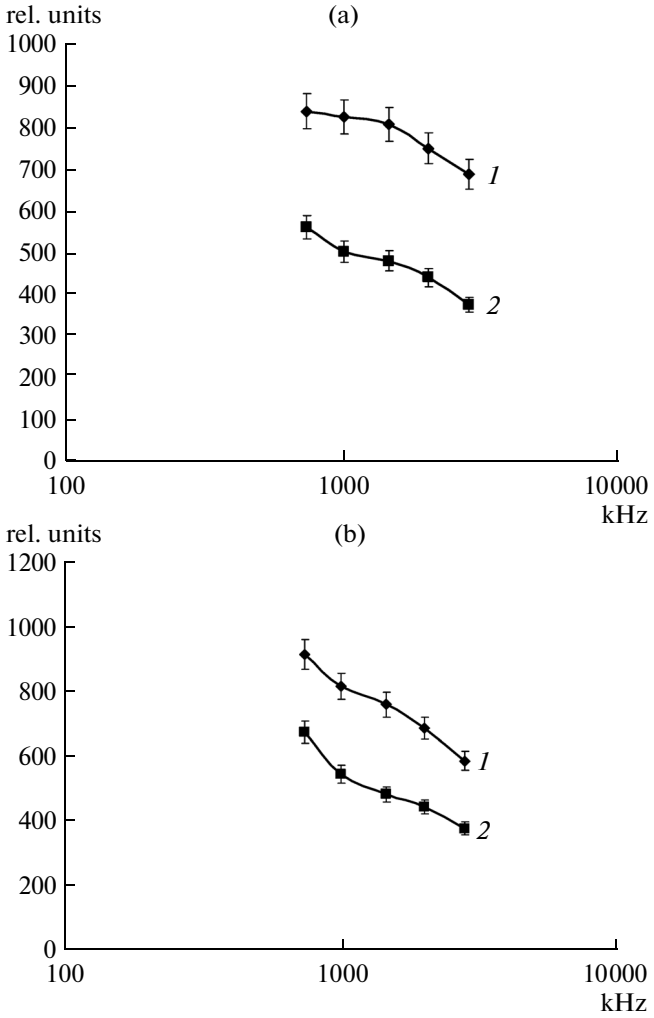

Fig. 2. Changes in the EO signal of an *A. brasilense* Sp7 suspension 10^6 (a) and 10^4 (b) cells/mL infected with the ΦAb-Sp bacteriophage. (*1*) Uninfected cells (control), (*2*) infected cells.

were infected with bacteriophage ΦAb-Sp7, which is probably due to the adsorption of bacteriophage on the cell surface. The value of this signal slightly varied when the bacteriophage effect was extended (Figs. 1a, 1b).

One of the important parameters of the detection method is the determination of the minimal number of detected cells. To determine this parameter, different numbers of cells (10^6, 10^4 and 10^2 cells/mL) were placed in the cell. An analytical signal registered after infection was not valid when the number of cells in the cell was 10^2 cells/mL; therefore, the results are not shown.

The results of OS measurements of *A. brasilense* Sp7cell suspensions containing 10^6 or 10^4 cells/mL after infection with bacteriophage ΦAb-Sp7 are shown in Fig. 2. The data demonstrated that the addition of the bacteriophage resulted in a significant change in the EO signal of the cell suspension. The limiting concentration of *A. brasilense* Sp7 cells should be 10^4 cells/mL in order to obtain reliable data by the EO analysis method.

A number of factors, primarily the presence of external microflora, complicates the generation of a reliable analytical signal in the analysis of cells by the EO method. Measurements of the EO parameters of the *A. brasilense* Sp7 cell suspension were made after infection with bacteriophage ΦAb-Sp7 in the presence of *Escherichia coli* cells strains B-878 and XL-1. The selection of these cell strains was determined by their differences in the taxonomic position and their size, which is close to that of *A. brasilense* Sp7. Bacteriophage cells (20 cells per bacteria) were added to the *A. brasilense* Sp7 suspension mixture (*E. coli* B-878 and *E. coli* XL-1, ratio 1 : 1 : 1, the number of cells in the measuring cell was 10^4 cells/mL) and incubated for 5 min. A mixture of *A. brasilense* Sp7, *E. coli* B-878, and *E. coli* XL-1 suspension without the addition of the bacteriophage was used as a control. We found a

significant reduction in the magnitude of the EO signal under these conditions (Fig. 3a). Our results showed that the presence of external cell cultures of *E. coli* strains B-878 and XL-1 did not complicate detection of *A. brasilense* Sp7 cells using bacteriophage ΦAb-Sp7. The study of nonspecific interaction of bacteriophage ΦAb-Sp7 with cells *E. coli* B-878 and *E. coli* XL-1 showed that the OS did not changed after infection. We can suggest that ΦAb-Sp7 bacteriophage did not infect the cells of *E. coli* strains B-878 (Fig. 3b) and XL-1 (Fig. 3c). This assumption was confirmed by the determination of the effect of the ΦAb-Sp7 bacteriophage on cultures of *E. coli* strains B-878 and XL-1 by the "drip" method [25]. The result was considered positive if a clear zone of lysis with secondary growth phagoresistant microorganisms was formed at the site of application of the bacteriophage on the continuous growth of the culture, and the result was considered negative in the absence of lysis.

It is known that some bacteriophages species have a broad spectrum of lytic activity and infect only certain bacterial strains of the same species, whereas other bacteriophage species are characterized by multiple virulence [26]. Therefore, we studied the selectivity of the investigated bacteriophage. According to the taxonomic position and relationship to the strain from which the bacteriophage was isolated, we tested the following bacterial species and strains: bacteria of the genus *Azospirillum*: *A. amazonense* Aml4; *A. brasilense* Cd, Spl07, Sp245, Jm6B2, Brl4, KR77, S17, S27, SR55, and SR75; *A. halopraeferans* Au4; *A. irakense* KBC1 and KA3; and *A. lipoferum* Sp59b, SR65, and RG20a.

The results of the study demonstrated that changes in the EO parameters of cell suspensions infected with ΦAb-Sp7 bacteriophage occurred only in *A. brasilense* cells of strains Sp7, Cd, SR55, Brl4, KR77, Spl07, and S27, as well as in *A. lipoferum* SR65, *A. amazonense* Aml4, *A. irakense* KBC1 and KA3, and *A. halopraeferans* Au4. At the same time, changes in the EO parameters were not detected in cell suspensions of the *A. brasilense* strains Sp245, SR75, S17, and Jm6B2 or in *A. lipoferum* Sp59b and RG20a. These results were confirmed by the standard microbiological method of determining the lytic activity of ΦAb-Sp7 bacteriophage by "dripping."

Thus, as a result of the study, the dependence of the EO parameters of *A. brasilense* Sp7 cell suspension after interaction with ΦAb-Sp7 bacteriophage on the number of cells and the time of contact was studied. We found that infection of the cells caused significant changes in the EO signal after one minute. The limit of the number of microbial cells required for a reliable estimate of the EO signal was determined. We studied the lytic activity of the ΦAb-Sp7 bacteriophage towards 18 strains of bacteria of the genus *Azospirillum*: *A. amazonense* Aml4, *A. brasilense* Sp7, Cd, Spl07, Sp245, Jm6B2, Brl4, KR77, S17, S27, SR55, SR75, *A. halopraeferans* Au4, *A. irakense* KBC1, KA3, *A. lipoferum* Sp59b, SR65 and RG20a.

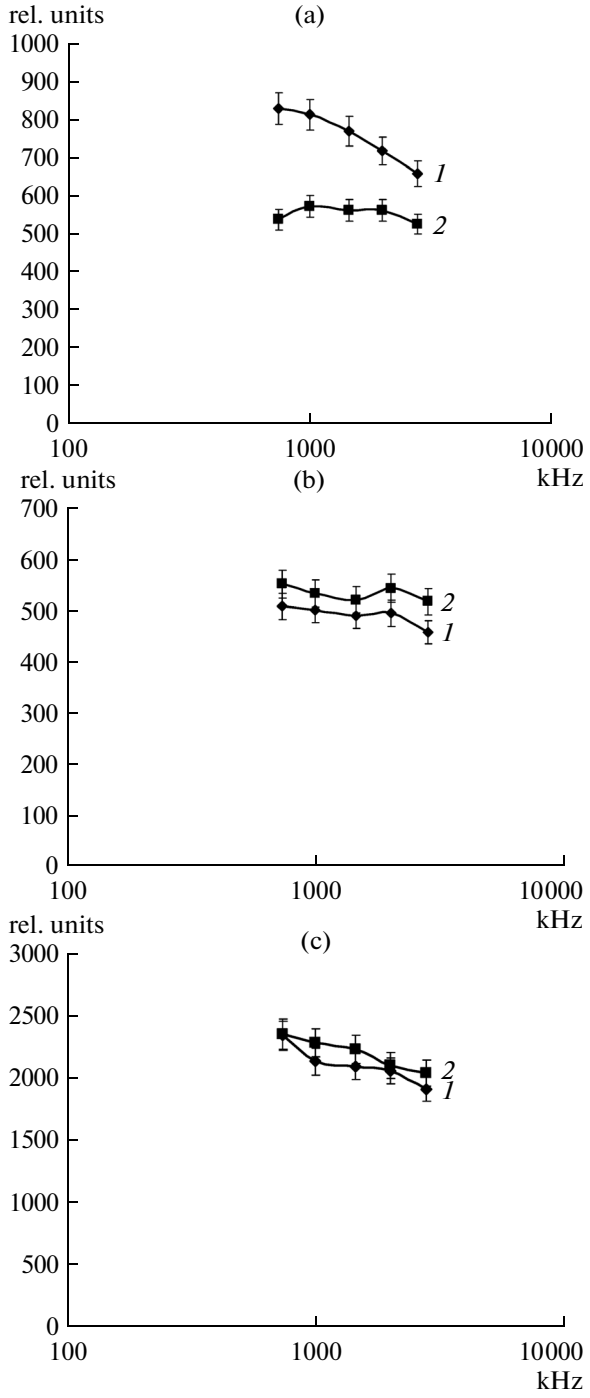

Fig. 3. The change in the EO signal after the addition of the ΦAb-Sp7 bacteriophage to *E. coli* B-878, *E. coli* XL-1 and *A. brasilense* Sp7 (a), *E. coli* B 878 (b) and *E. coli* XL-1 (c) cell mixture. (*1*) Uninfected cells (control), (*2*) infected cells.

These results demonstrate the possibility of using ΦAb-Sp7 bacteriophage for detecting microbial *Azospirillum* cells by electrooptical analysis of cell sus-

pensions. This approach can be used to develop methods for express detection of *Azospirillum* cells.

REFERENCES

1. Steenhoudt, O. and Vanderleyden, J., *FEMS Microbiol. Lett.*, 2000, vol. 24, no. 4, pp. 487–506.
2. Bashan, Y., *Can. J. Microbiol.*, 1998, vol. 44, no. 2, pp. 168–174.
3. *Molekulyarnye osnovy vzaimodeistviya assotsiativnykh mikroorganizmov s rasteniyami* (Molecular Basis of Interaction of Associative Microorganisms with Plants), Ignatov, V.V., Ed., Moscow: Nauka, 2005.
4. Baldani, J.I. and Baldani, V.L., *Acad. Bras. Cienc.*, 2005, vol. 77, no. 3, pp. 549–579.
5. Mulyukin, A.L., Suzina, N.E., Pogorelova, A.Yu., Antonyuk, L.P., Duda, V.I., and El'-Registan, G.I., *Microbiology* (Moscow), 2009, vol. 78, no. 1, pp. 33–41.
6. Krasov, A.I., Popova, I.A., Filip'echeva, Yu.A., Burygin, G.L., and Matora, L.Yu., *Microbiology* (Moscow), 2009, vol. 78, no. 5, pp. 598–602.
7. Schmelcher, M. and Loessner, M., in *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems*, Zourob, M., Elwary, S., and Turner, A., Eds., New York: Springer Science, Business Media, LLC, 2008, pp. 731–754.
8. Ripp, S., *Adv. Biochem. Engin. Biotechnol.*, 2010, vol. 118, pp. 65–84.
9. Singh, A., Arutyunov, D., Szymanski, C.M., and Evoy, S., *Analyst*, 2012, vol. 137, no. 15, pp. 3405–3421.
10. Vaughan, R.D., O'Sullivan, C.K., and Cuilbault, G.G., *Enzyme Microb. Technol.*, 2001, vol. 29, no. 10, pp. 635–638.
11. Zaitsev, B.D., Kuznetsova, I.E., Shikhabudinov, A.M., Ignatov, O.V., and Guliy, O.I., *IEEE Trans. Ultrason Ferroelectr. Freq. Control*, 2012, vol. 59, no. 5, pp. 963–969.
12. Smartt, A.E. and Ripp, S., *Anal. Bioanal. Chem.,* 2011, vol. 400, no. 4, pp. 991–1007.
13. Ignatov, O.V., Guliy, O.I., and Bunin, V.D., in *Commercial and Pre-commercial Cell Detection Technologies for Defense against Bioterror—Technology, Market and Society*, Lechuga, L.M., Milanovich, F.P., Skladal, P., Ignatov, O.V., and Austin, T., Eds., Amsterdam: IOS Press, 2008, pp. 45–53.
14. Gulii, O.I., Matora, L.Yu., Burygin, G.L., Dykman, L.A., Ignatov, V.V., and Ignatov, O.V., *Appl. Biochem. Microbiol.*, 2010, vol. 46, no. 1, pp. 61–64.
15. Shchyogolev, S.Y., Khlebtsov, N.G., Bunin, V.D., Sirota, A.I., and Bogatyrev, V.A., in *Inverse Problems of Spectroturbidimetry of Biological Disperse Systems with Random and Ordered Particle Orientation*, Chance, B., Delpy, D.T., Ferrari, M., van Gemert, M.J. C., Mueller, G.J., and Tuchin, V.V., Eds., Bellingham, Washington: SPIE, 1994, pp. 167–176.
16. Guliy, O.I., Matora, L.Yu., Burygin, G.L., Dykman, L.A., Ostudin, N.A., Bunin, V.D., Ignatov, V.V., and Ignatov, O.V., *Anal. Biochem.*, 2007, vol. 370, no. 2, pp. 201–205.
17. Gulii, O.I., Bunin, V.D., Neil, D., Ivnitskii, D., Matora, L.Yu., Ignatov, V.V., and Ignatov, O.V., *Zh. Mikrobiol. Epidemiol. Immunol.*, 2007, no. 5, pp. 80–82.
18. Bunin, V.D., Ignatov, O.V., Gulii, O.I., Zaitseva, I.S., Neil, D., and Ivnitskii, D., *Microbiology* (Moscow), 2005, vol. 74, no. 2, pp. 164–168.
19. Sambrook, J., in *Molecular Cloning: A Laboratory Manual*, Sambrook, J., Fritsch, E.F., Maniatis, T., Eds., New York: Cold Spring Harbor Laboratory Press, 1982.
20. Ignatov, O.V., Guliy, O.I., Shchyogolev, S.Yu., Bunin, V.D., and Ignatov, V.V., *FEMS Microbiol. Letts.*, 2002, vol. 214, no. 1, pp. 81–86.
21. Elmerich, C., Quiviger, B., Rosenberg, C., Franche, C., Laurent, P., and Döbereiner, J., *Virology*, 1982, vol. 122, no. 1, pp. 29–37.
22. MC, GreenP., *Genome Res.*, 1998, vol. 8, no. 3, pp. 175–185.
23. Germida, J.J., *Can. J. Microbiol.*, 1984, vol. 30, no. 6, pp. 805–808.
24. Boyer, M., Haurat, J., Samain, S., Segurens, B., Gavory, F., González, V., Mavingui, P., Rohr, R., Bally, R., and Wisniewski-Dyé, F., *Appl. Environ. Microbiol.*, 2008, vol. 74, no. 3, pp. 861–874.
25. Adams, M., *Bakteriofagi* (Bacteriophages), Adams, M., Ed., Moscow: Medgiz, 1961.
26. Labinskaya, A.S., *Mikrobiologiya s tekhnikoi mikrobiologicheskikh issledovanii* (Microbiology with the Technique of Microbiological Tests), Efremova, S.A, Ed., Moscow: Meditsina, 1978.

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