ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 3, pp. 334–338. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © O.I. Guliy, L.N. Markina, V.D. Bunin, V.V. Ignatov, O.V. Ignatov, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 3, pp. 380–385.

EXPERIMENTAL ARTICLES

Electrooptical Parameters of Kanamycin-Treated *E. coli* Cell Suspensions

O. I. Guliy[a], L. N. Markina[a], V. D. Bunin[b], V. V. Ignatov[a], and O. V. Ignatov[a,1]

[a] *Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov, 13, 410049 Saratov, Russia*

[b] *State Scientific Center for Applied Microbiology, Obolensk, Moscow oblast*

Received March 26, 2007

Abstract—The effect of kanamycin on the electrophysical parameters of cell suspensions of *Escherichia coli* K-12 and pMMB33 was investigated. Incubation of the sensitive K-12 strain with kanamycin resulted in significant changes in the orientation spectra (OS) of the cell suspensions; these changes were not revealed in the case of the resistant pMMB33 strain. In the case of the sensitive K-12 strain incubated with different kanamycin concentrations, changes in the OS of the cell suspensions occurred within the 10–1000 kHz frequency range of the orienting electrical field. The most pronounced change in the electrooptical signal was observed at 10 μg/ml of kanamycin. Control experiments were carried out by standard plating on nutrient media. Thus, the OS changes of suspensions in the presence of antibiotics may be used as a test for microbial resistance to such antibiotics.

Key words: Escherichia coli, orientation spectra, kanamycin.

DOI: 10.1134/S0026261708030132

Since the emergence of microorganisms hinders the therapeutic application of antibiotics, investigation of microbial adaptation to antibiotics is an important biomedical problem. The effect of antibiotics may be due to a variety of factors, including the inhibition of cell wall synthesis, synthesis of proteins and/or RNA, and DNA replication, as well as impairment of the functioning of membranes [1]. In the presence of antibiotics, the morphology of microbial cells may be changed, their cellular membrane disrupted, and their cytoplasmic membrane modified; the biochemical processes occurring in these structures may therefore be impaired. This, in turn, may result in altered electrophysical (EP) characteristics of microbial cells and therefore in altered electrooptical (EO) characteristics of cell suspensions, which can be experimentally detected using the electrical orientation of the cells in an electric field. We have previously demonstrated the possibility of determining microbial resistance to ampicillin [2]. We believe that EO analysis of cell suspensions in the presence of antibiotics with an action mechanism different from that of ampicillin is a promising area of research. Inhibitors of protein synthesis, including aminoglycoside antibiotics, are an example. These antibiotics penetrate the outer membrane of gram-negative bacteria and displace magnesium ions from the external surface of the outer membrane; this process results in partial destruction of the membrane [3–4]. We expected that the membrane destruction caused by an antibiotic could change the EP characteristics of microbial cells; these, in turn, could be used as an indicator of microbial sensitivity to antibiotics under study.

The goal of the present work was to investigate the effect of kanamycin on the EO parameters of the cell suspensions of some *Escherichia coli* strains with different sensitivity to the antibiotic in question.

MATERIALS AND METHODS

Microorganisms. Strains *E. coli* K-12 and *E. coli* pMMB33 used in the present work were obtained from the strain collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov, Russia).

E. coli* K-12 and *E. coli pMMB33 were grown in a liquid medium containing the following (g/l): NaCl, 10; yeast extract, 5; and peptone, 5. The cultivation was carried out under aerobic conditions on a rotary shaker (160 rpm) at 30°C for 24 h. The cells were collected and used for EO studies.

[1] Corresponding author; e-mail: oignatov@ibppm.sgu.ru

Cell preparation for analysis. Prior to analysis, the cells were washed three times with 5-min centrifugation at 2800 *g* and resuspended in a small volume of distilled water (conductivity 1.8 μS/cm). To remove cell aggregates, the suspension was centrifuged for 1 min at 110 *g*; the supernatant was collected for the experiments. For every microbial strain, OD_{670} was adjusted to 0.4–0.42.

Orientation spectra of the cells were measured on an ELBIC electrooptical analyzer (State Scientific Center for Applied Microbiology, Obolensk, Moscow oblast) at 670 nm (relative to vacuum) according to [5]. The set of frequencies for the orienting electric field was as follows: 10, 52, 104, 502, 1000, 5020, and 10000 kHz. The orientation spectrum (OS) was represented as a frequency function of the difference between the OD values determined in nonpolarized light beams directed along and across the orienting field (δOD). This difference was normalized to the optical density obtained for chaotically oriented cells [6–8].

Microbial sensitivity to antibiotics was determined by the serial dilutions method [9]. The amount of an antibiotic in the test tube with visible growth inhibition was accepted as the minimal inhibiting concentration for the strain in question.

Antibiotic treatment. After the 24-h cultures of *E. coli* K-12 and *E. coli* pMMB33 were prepared for EO analysis, kanamycin (Sigma, United States) was introduced into the cell suspension (OD_{670} = 0.4–0.45). The suspension was incubated at 30°C for 5, 15, 30, 60, or 150 min. The cell suspension incubated for the same time at the same temperature without antibiotics was used as a control. After incubation, the cells were washed three times with distilled water (1.6–2.0 μS/cm) and used for EO measurements.

Colony count. In order to determine the number of colonies formed from individual viable cells, the standard plating method was used. The agarized medium contained the following (g/l): NaCl, 10; yeast extract (Fluka, Switzerland), 5; peptone (Fluka, Switzerland), 5. The cell suspension prepared for EO analysis was treated with kanamycin and incubated for 30 min at 37°C. The diluted suspension (0.1 ml) was applied to the surface of dried agar in petri dishes and spread with a glass spreading rod. After overnight incubation at 30°C, the number of colonies was determined under adequate illumination. The colony counts obtained without antibiotic treatment were used as the control [10].

RESULTS AND DISCUSSION

Kanamycin is an aminoglycoside antibiotic of the oligosaccharide group. It operates mainly by disrupting protein synthesis at the stage of amino acid transfer from aminoacyl-tRNA to the ribosome. Kanamycin promotes binding with the ribosomes of those aminoacyl-tRNAs that do not correspond to the codon of the ribosomal A site. Due to such faulty encoding, polypeptides with numerous errors are synthesized; a cytotoxic (bactericidal) effect results [11]. Since this antibiotic is active against various gram-negative rods, *E. coli* was chosen as an object of our study. The experiment was aimed at comparative study of electrooptical characteristics of microbial cells with different sensitivity to an antibiotic.

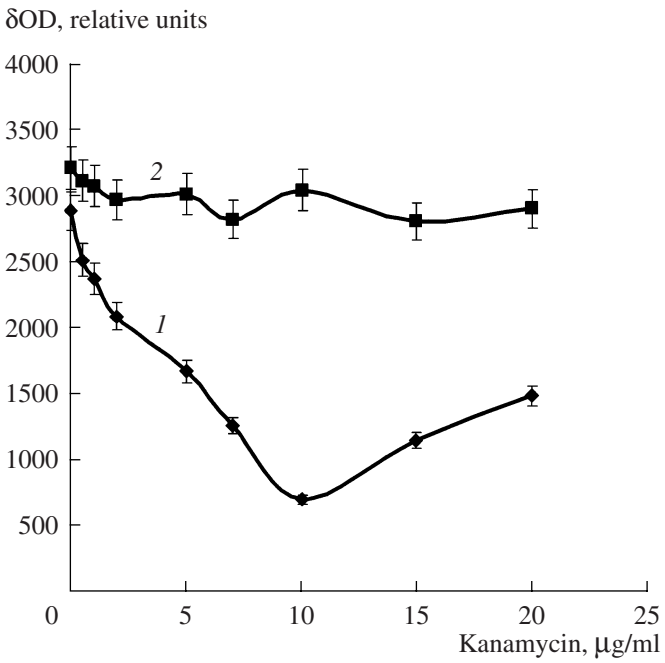

Fig. 1. Dynamics of the changes in the EO signal at a 52 kHz orienting field frequency for *E. coli* K-12 (*1*) and resistant strain *E. coli* pMMB33 (*2*) incubated with different kanamycin concentrations (0.5, 1.0, 2.0, 5.0, 7.0, 10, 15, and 20 μg/ml).

At the first stage, the EO characteristics of the cell suspension of the kanamycin-sensitive strain *E. coli* K-12 was studied under different kanamycin concentrations (0.5, 1.0, 2.0, 5.0, 7.0, 10, 15, and 20 μg/ml). The changes in OS of the K-12 cell suspensions were revealed at frequencies of the orienting electric field within the range of 10–1000 kHz. No considerable changes were detected at higher frequencies. For more convenient representation of the experimental data, we present the EO signal obtained at an orienting field of 52 kHz. The data presented in Fig. 1 (curve *1*) demonstrate that addition of the above antibiotic concentrations resulted in a gradual decrease in the EO signal, with the minimal value at 10 μg/ml kanamycin. The mechanism of antimicrobial action of kanamycin is related to suppressed protein synthesis with the subsequent inhibition of nucleic acid synthesis and disrupted cell wall formation. Aminoglycosides penetrate the outer membrane of gram-negative bacteria via porin channels; the membrane is partially disrupted, and ami-

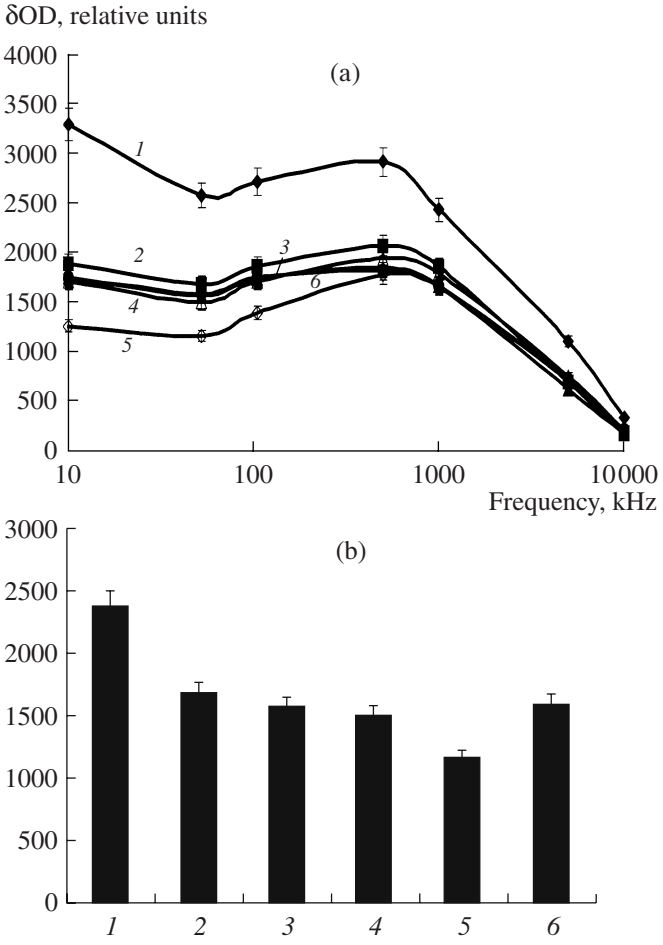

Fig. 2. Dynamics of changes of the OS (a) and the EO signal (b) at 52 kHz orienting field frequency for *E. coli* K-12 cell suspensions incubated with kanamycin (10 μg/ml): control without kanamycin (*1*); 5 min (*2*); 15 min (*3*); 30 min (*4*); 60 min (*5*); and 150 min (*6*).

noglycoside penetration through this barrier intensifies [4]. Therefore, a decreased EO signal at a kanamycin concentration of 10 μg/ml is possibly due to disruption of the cell membrane and to bactericidal action of the antibiotic against *E. coli* K-12 cells.

The mechanism of kanamycin resistance involves enzymatic inactivation of the antibiotic via acetylation of its amino group or phosphorylation of its hydroxyl group; it is determined by a transmissible R factor. Modification of kanamycin by bacterial enzymes results in its loss of antibiotic activity [1, 12]. At the next stage of investigation, the EO parameters of the suspension of kanamycin-resistant *E. coli* pMMB33 cells were studied under different kanamycin concentrations (0.5, 1.0, 2.0, 5.0, 7.0, 10, 15, and 20 μg/ml). This strain carries the pMMB33 plasmid providing resistance to kanamycin. No significant OS changes were revealed in the cell suspension treated with the antibiotic (Fig. 1, curve *2*). These results may be explained by kanamycin inactivation by the R enzymes phosphorylating kanamycin and thus depriving it of activity. This assumption correlates with the published data on the genetic information concerning synthesis of R enzymes encoded by genes localized on the chromosome, plasmids, or episomes [12]. In our experiments, the kanamycin-resistant strain *E. coli* pMMB33 was used; this strain carries the pMMB33 plasmid providing resistance to kanamycin. Genetic information concerning synthesis of R enzymes in this strain is probably controlled by the pMMB33 plasmid.

Comparison of the experimental results on EO parameterchanges in the kanamycin-sensitive and resistant strains (Fig. 1) demonstrates that in the sensitive *E. coli* K-12 cells, the EO signal changed with the most pronounced decrease in the EO signal at 10 μg/ml kanamycin. No significant changes in the EO signal were revealed in the resistant strain *E. coli* pMMB33.

At the next stage, the dynamics of the OS changes was studied in these strains in the presence of kanamycin (10 μg/ml). Figure 2 demonstrates that in the case of the sensitive strain *E. coli* K-12, a decrease in the EO signal value was detected already after 5 min of antibiotic treatment. This is possibly the result of partial destruction of the cell membrane and antibiotic penetration into the cell [13]. After 15 min of incubation with kanamycin, the EO signal continued to decrease gradually. These changes of the EO signal value may be linked to the biochemical processes occurring in the cell affected by kanamycin and to increased antibiotic penetration into the cell. Monitoring the changes of the EO parameters of *E. coli* K-12 cell suspension incubated with kanamycin may therefore be an indictor of antibiotic penetration into the cell and the sensitivity to kanamycin. Thus, bactericidal action of antibiotic against *E. coli* K-12 cells was detected at a kanamycin concentration of 10 μg/ml.

The dynamics of the changes in the EO parameters of the cell suspension of kanamycin-resistant *E. coli* pMMB33 incubated with kanamycin revealed an insignificant increase of the EO signal after 5-min incubation with kanamycin (Fig. 3). No significant OS changes of the cell suspension were revealed in the course of a more prolonged cell incubation with kanamycin; this is possibly an indication of the strain's resistance to kanamycin.

Since we believe the changes in the EO signal at high antibiotic concentrations to be related to its bactericidal action, experiments on cell viability were performed. The standard method of colony counting after treatment with kanamycin (7, 10, 15, an 20 μg/ml) was used. It was shown (table) that the number of viable cells decreased significantly after treatment with high concentrations of kanamycin. Simultaneous experiments with kanamycin-treated cells of *E. coli* pMMB33 revealed almost no changes in the number of viable cells after kanamycin treatment (Table). However, the

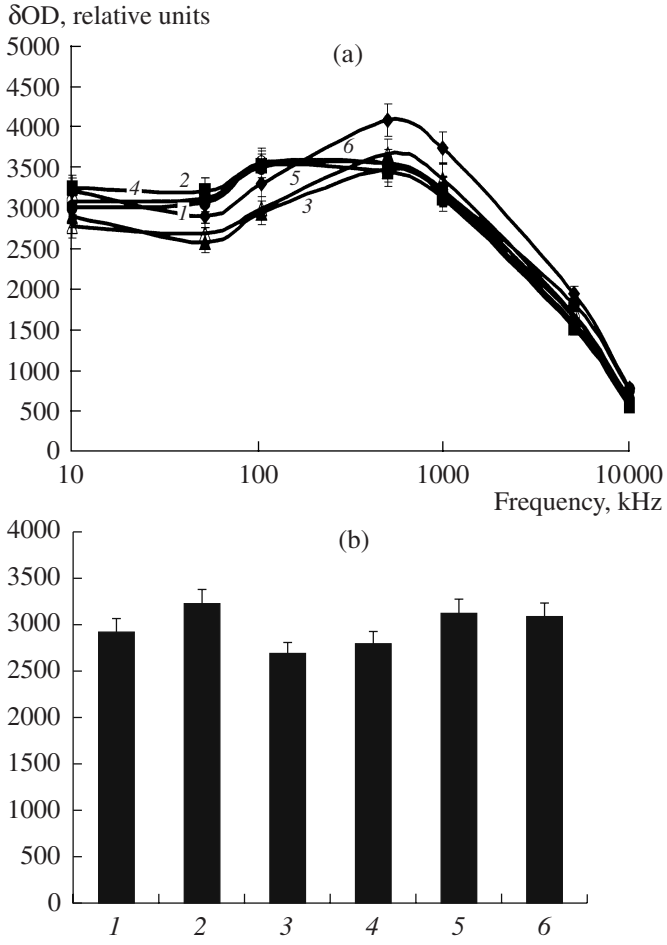

Fig. 3. Dynamics of changes of the OS (a) and the EO signal (b) at 52 kHz orienting field frequency for *E. coli* pMMB33 cell suspensions incubated with kanamycin (10 μg/ml): control without kanamycin (*1*); 5 min (*2*); 15 min (*3*); 30 min (*4*); 60 min (*5*); and 150 min (*6*).

number of viable cells decreased when a kanamycin concentration of 20 μg/ml was applied.

Thus, our research revealed that the EO effect observed in kanamycin-treated microbial cells was substantially different for sensitive and resistant *E. coli* cells. Kanamycin-induced OS changes in cell suspensions may be used to test bacterial resistance to the antibiotic under study.

The presently accepted methods for determining microbial sensitivity to antibiotics include standard disk methods, serial dilution methods, and modifications of these standard procedures [10]. Automated systems for determination of antibiotic sensitivity have been developed. In some of them, only dilution and incubation are automatic, while bacterial growth is determined by traditional methods. In others, all the initial operations are performed manually and only the reading and detection of results is automatic. Some systems require programming of all of the operations performed in the course of analysis (sample and inoculum preparation, incubation, reading and registration of results) [1]. Analysis of such modern technologies for determining antibiotic-sensitive bacteria as microbial biosensor systems [14–16] and the BACTEC radiometric method [17] demonstrates that the basic problems with the given methods are sample collection and preparation, elimination of false positive results, and a long time of measurement. The development of new technologies and methods for determining bacterial sensitivity to antimicrobial agents is therefore important for biology and medicine. Our results suggest that EO analysis of cell suspensions for determining bacterial resistance to antibiotics is a promising field of research. Unlike the standard methods, EO analysis has such advantages as rapidity of results and simplicity of the analytical procedure; moreover, minimal volumes are required for analyses.

ACKNOWLEDGMENTS

The work was supported by the President of the Russian Federation Foundation for Promotion of Rus-

Determination of viability (V.) of *E. coli* K-12 and *E. coli* pMMB33 cells by plating on agarized media

Control: cells not treated with kanamycin		Cells after kanamycin treatment							
		7 μg/ml		10 μg/ml		15 μg/ml		20 μg/ml	
E. coli K-12									
Colony number, $\times 10^8$	V., %	Colony number, $\times 10^8$	V., %	Colony number, $\times 10^8$	V., %	Colony number, $\times 10^5$	V., %	Colony number, $\times 10^5$	V., %
107 ± 0.5	100	41 ± 0.9	38	20 ± 0.7	18	17 ± 0.4	16	12 ± 1	11
E.coli pMMB-33									
Colony number, $\times 10^8$	V., %	Colony number, $\times 10^8$	V., %	Colony number, $\times 10^8$	V., %	Colony number, $\times 10^8$	V., %	Colony number, $\times 10^8$	V., %
67 ± 0.8	100	66 ± 0.7	99	67 ± 0.4	100	63	94	56	84

sian Science for Young Candidates of Sciences (project no. MK-8599.2006.4), the Program of State Support of the Leading Scientific Schools of the Russian Federation (project no. NSh-6177.2006.4), and the Youth Science and Technology Center (project no. 3170 PDG).

REFERENCES

1. Lanchini, D. and Parenti, F., *Antibiotiki* (Antibiotics), Moscow: Mir, 1985.
2. Gulii, O.I., Markina, L.N., Ignatov, O.V., Shchegolev, S.Yu., Bunin, V.D., and Ignatov, V.V., The Effect of Ampicillin on the Electrophysical Properties of *Escherichia coli* Cells, *Mikrobiologiya,* 2005, vol. 74, no. 1, pp. 126–131 [*Microbiology* (Engl. Transl.), vol. 74, no.1, pp. 111–115].
3. Brian, L.E., *Bakterial'naya rezistentnost' i chuvstvitel'nost' k khimiopreparatam* (Bacterial Resistence and Sensitivity to Chemical Preparations), Moscow: Meditsina, 1984.
4. Sazykin, Yu.O. and Navashin, P.S., Antibiotics and Bacterial Cell Envelope, *Itogi nauki i tekhniki. VINITI. Ser. Biotekhnol.*, Moscow, 1991, vol. 31, pp. 1–187.
5. Miroshnikov, A.I., Fomchenkov, V.M., and Ivanov, A.Yu., *Elektofizicheskii analiz i razdelenie kletok* (Electrophysical Analysis and Separation of Cells), Moscow: Nauka, 1986.
6. Ignatov, O.V., Guliy, O.I., Shchyogolev, S.Yu., Bunin, V.D., and Ignatov, V.V., Effect of *p*-Nitrophenol Metabolites on Microbial-Cell Electro-Optical Characteristics, *FEMS Microbiol. Letts.*, 2002, vol. 214, pp. 81–86.
7. Bunin, V.D. and Voloshin, A.G., Determination of Cell Structures, Electrophysical Parameters, and Cell Population Heterogeneity, *J. Colloid Interface Sci.*, 1996, vol. 180, pp. 122–126.
8. Bunin, V.D., Voloshin, A.G., Bunin, Z.F., and Shmelev, V.A., Electrophysical Monitoring of Culture Process of Recombinant *Escherichia coli* Strains, *Biotechnol. Bioeng.*, 1996, vol. 51, pp. 720–724.
9. Labinskaya, A.S., *Mikrobiologiya s tekhnikoi mikrobiologicheskikh issledovanii* (Microbiology and Techniques for Microbiological Investigation), Moscow: Meditsina, 1978.
10. *Manual of Methods for General Bacteriology*, Gerhardt, P. et al., Eds., Washington: Amer. Soc. Microbiol., 1981 [Russ. Transl. Moscow: Mir, 1984, vol. 1, pp. 458–464].
11. Spirin, A.S., *Molekulyarnaya biologiya. Struktura ribosomy i biosintez belka* (Molecular Biology. Ribosome Structure and Protein Synthesis), Moscow: Vysshaya shkola, 1986.
12. Kudlai, D.G., Chubukov, V., and Oganesyan, M., *Genetika lekarstvennoi ustoichivosti bakterii* (Genetics of Bacterial Drug Resistance), Moscow: Meditsina, 1972.
13. Egorov, N.S., *Osnovy Ucheniya ob Antibiotikakh* (Basic Principles of the Theory of Antibiotics), Moscow: Vysshaya Shkola, 1986.
14. Galindo, E., Bautista, D., Garcia, J.L., and Quintero, R., Microbial Sensor for Penicillins Using a Recombinant Strain of *Escherichia coli, Enz. Microb. Technol.*, 1990, vol. 12, pp. 642–646.
15. Galindo, E., Lagunas, F., Osuna, J., Soberon, X., and Garcia, J.L., A Microbial Biosensor for 6-Aminopenicillanic Acid, *Enz. Microb. Technol.*, 1998, vol. 23, pp. 331–334.
16. Fleschin, S., Bala, C., Bunaciu, A.A., Panait, A., and Aboulenein, H.Y., Enalapril Microbial Biosensor, *Prepar. Biochem. Biotechnol.*, 1998, vol. 28, pp. 261–269.
17. Cavalieri, S.J., Biehle, J.R., and Sanders, W.E., Jr., Synergistic Activities of Clarithromycin and Antituberculous Drugs Against Multidrug-Resistant *Mycobacterium tuberculosis, Antimicrob. Agents Chemother.*, 1995, vol. 39, no. 7, pp. 1542–1545.