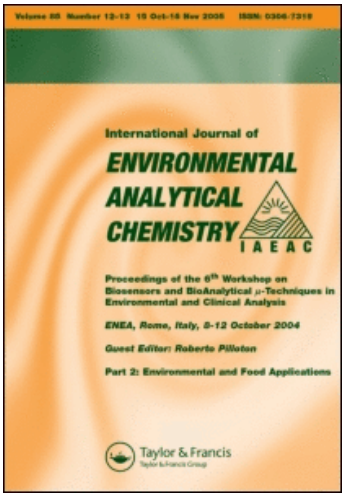


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# Action of ampicillin and kanamicin on the electrophysical characteristics of *Escherichia coli* cells

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We examined the effect of ampicillin and kanamycin on the electrophysical characteristics of ampicillin- and kanamycin-sensitive and ampicillin- and kanamycin-resistant *Escherichia coli* cells. Substantial changes in the orientational spectra (OS) of suspensions of cells incubated with various ampicillin and kanamycin concentrations took place only at the first five frequencies of the orienting electric field (10–1000 kHz). The maximal change in the magnitude of the electro-optical signal occurred at $50\,\mu g\,mL^{-1}$ of ampicillin and $10\,\mu g\,mL^{-1}$ of kanamycin. The suspension-OS changes did not depend on the antibiotic-action period. Under the action of ampicillin and kanamycin, sensitive and resistant *E. coli* strains gave different electro-optical (EO) effects. It follows that the sensitive and resistant *E. coli* strains exhibit different of the EO effect on the action of ampicillin and kanamycin. Thus, the suspension-OS changes occurring under the effect of ampicillin and kanamycin may be used as a test for resistance to this antibiotic in the cells studied.

*Keywords:* *Escherichia coli*; Electroorientation; Orientational spectrum; Ampicillin; Plasmid

## 1. Introduction

Study of the adaptation of microbes to antibiotic action is an important problem that is of theoretical and applied significance. There are different approaches for investigation of antibiotic resistance [1, 2].

Ampicillin is a $\beta$-lactamic antibiotic that is produced by aminophenylacetic acid acetylation of 6-aminopenicillanic acid. It is a broad-spectrum agent that is active specifically toward *E. coli*.

It is known that the action of amoxicillin on ampicillin-sensitive microbes results in changes in the character of biochemical processes and metabolic pathways, as well as in cell morphology (cell lengthening and swelling, their bending, chain or ball

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formation, lysis) [3]. The action mechanism of $\beta$-lactamic antibiotics is associated with a disturbance in cell-wall formation in bacteria – more specifically, with a disturbance in the synthesis of its main polymer, peptidoglycan. As a result of antibiotic action on the enzyme catalysing the reaction of transpeptidation, this reaction is inhibited, and the formation of peptide bridges between parallel glycan threads stops [3, 4].

Kanamycin, an inhibitor of protein synthesis, is an aminoglycaside antibiotic of the oligosaccharide family. This antibiotic was chosen because, in contrast to other bacteriostatic antibiotics that inhibit protein synthesis, it is weakly bactericidal. It has entered microbial cells through porin channels, kanamycin suppresses protein synthesis and does not cause changes in cellular morphological characteristics.

One can assume that changes in cell morphology and cell-wall disturbances in antibiotic-resistant microorganisms should lead to changes in their electrophysical characteristics. These changes are reflected in alterations in the electro-optical characteristics of cell suspensions, which are recorded during experiments using electric-field cell orientation. On the basis of these alterations, one can draw tentative conclusions about the presence or absence of resistance to a given antibiotic in the cells under study.

The aim of this work was to study the effect of ampicillin and kanamycin on the EO parameters of cell suspensions of *E. coli* strains differing in their resistance to this antibiotic.



## 2. Experimental

### 2.1 *Microorganisms*

*Escherichia coli* K-12, *E. coli* XL-1, *E. coli* K-12 (pUC-18), and *E. coli* K-12 (PMMB33) were received from the collection at the Institute of Biochemistry and Physiology of Plants and Microorganisms, Saratov.

### 2.2 *Culture conditions*

*Escherichia coli* K-12, *E. coli* XL-1, *E. coli* K-12 (pUC-18), and *E. coli* K-12 (PMMB-33) was grown in liquid medium containing ($g\,L^{-1}$): NaCl, 10; yeast extract (FLUKA, Switzerland), 5; peptone (FLUKA, Switzerland), 5. The culture was shaken (160 rpm) aerobically for 24 h at a constant temperature of 30°C. The cells grown were used for EO studies.

### 2.3 *Measurement of cellular orientational spectra*

The orientational spectra (OS) of the cells were measured with an ELBIC EO analyser at a wavelength of 670 nm. An OS is given as the frequency dependence of the difference ($\delta D$) between the suspension-optical-density values ($D_a$ and $D_b$) measured during the propagation of a beam of non-polarized light along ($D_a$) and across ($D_b$) the orienting-field direction. This difference was normalized to the optical density value ($D$) measured for cells at random orientation. There is reason to assume that the general view of the OS under the chosen experimental conditions (wavelength, relatively

small amplitude of the orienting-electric-field strength, etc.) is essentially determined by the frequency dependence of the anisotropy of the cell polarizability $\Delta\gamma = \gamma_a - \gamma_b$ [5, 6]:

$$\delta D(\omega) = (D_a - D_b)/D = \Delta\gamma(\omega)E^2F \tag{1}$$

where $E$ is the orienting-field strength, $\omega$ is the orienting-field frequency, and $F$ is a coefficient including an 'optical factor', which depends, among other things, on the cell size and refractive index.

For rod-shaped axisymmetric particles, which are good models for the cell types used by us, the polarizability tensor has only two distinct components [7]: a longitudinal one, $\gamma_a$ (corresponding to the direction of the long axis of the particle) and a transverse one, $\gamma_b$ (corresponding to the orthogonal direction). The preferred direction (along or across the strength-vector direction of the orienting electric field) and the degree of particle orientation (characterized by the width of the Boltzmann function of particle distribution by orientational angle) depend on the sign of $\Delta\gamma$ and on the value of the parameter:

$$q = \Delta\gamma E^2/(2kT) \tag{2}$$

where $k$ is the Boltzmann constant and $T$ is the absolute temperature. To the relatively weak cell-orientation degree in our experiment there corresponds the range of value $q \ll 1$.

The design of the ELBIC apparatus makes it possible to represent the OS as the frequency dependence of the relation:

$$\delta OD_{\text{relative units}} = \delta D/(E^2F\kappa) \approx A\Delta\gamma \tag{3}$$

which was used in our experiments. In this equation, $\kappa = I/I_0$ is the transmittance coefficient ($I$ and $I_0$ are the intensities of transmitted and incident light, respectively). The constant $A$ contains a scale multiplier that makes for easy reading of the measured results for $\delta OD_{\text{relative units}}$, the magnitude of which in this case had values of the order $10^2$–$10^3$. At sufficiently low degrees of cell orientation, such normalization ensures the independence of $\delta OD_{\text{relative units}}$ from the cell concentration, the orienting-field strength $E$, and the attenuation of the light beam during its passage through the scattering medium.

Thus, the dependence of $\delta OD_{\text{relative units}}$ on the orienting-field frequency $\omega$ coincides with the frequency dispersion of the anisotropy of the particle-polarizability tensor $\Delta\gamma(\omega)$ with an accuracy of the constant. Depending on the orienting-field frequency, the frequency dispersion $\Delta\gamma(\omega)$ mirrors the effects of various cell structural elements. These elements are: (i) cell-surface biopolymers and associated high- and low-molecular-weight compounds (coming from the environment) that form a double electric layer directly at the cell–environment interface ($\omega$ of the order of units and tens of Hz); (ii) the components of the cell wall and cytoplasmic membrane ($\omega$ of the order of tens and hundreds of kHz); and (iii) the elements of the cell inner structure (cell organelles) ($\omega$ of the order of units and tens of MHz).

This makes it possible to obtain information on the various physical–chemical and physiological–biochemical processes occurring on the surface of and inside the cell, including substrate-induced enzymatic processes taking place in microbial cells.

The analysis conditions were: volume of the measuring cell, 1 mL; cell concentration (in optical-density units) $D_{670} = 0.45$–$0.50$. To this range of $D_{670}$ values there corresponds a cell concentration of about $10^9\,mL^{-1}$ for all the test strains (conductivity of the dispersion medium, 1.6–2.0 $S\,m^{-1}$). At such a cell concentration, the cell-to-cell distance is, on the average, more than 100 times greater than the cell size. This fact almost rules out the field-induced formation of cell chains because, as shown by special experiments, such formation requires higher cell concentrations, with the cell-to-cell distance being commensurable with the cell size.

Cells to be used in the analysis were washed three times by centrifugation at $5000\,g$ for 5 min and resuspended in distilled water to an optical density ($D_{670}$) of 0.5. To remove cellular aggregates, we re-centrifuged the cell suspension at $1000\,g$ for 1 min, and further work was carried out using the suspension that remained in the supernatant liquid. The cells thus prepared were incubated with ampicillin for different times at 30°C and subjected to EO measurements. Within this time interval, the recorded changes in the OS became stable, and no additional changes were observed. A discrete set of frequencies of the orienting electric field (10, 52, 104, 502, 1000, 5020, and 10,000 kHz) was used.



### 2.4 *Phage infection*

In this work, we used plasmid pHEN1 which is constructed on the basis of the genome M13K07. M13K07, is a filamentous phage of the family *Inoviridae*. M13K07, a kanamycin-resistant commercial preparation manufactured by Stratagene (Sweden), was constructed on the basis of the wide-type phage M13 [8, 9].

For transfection, *E. coli* XL-1 Blue *recA1 endA1 gyrA96 thi*-1 *hsdR17 supE44 relA1 lac*[F'*proABlacI*$^q$*Z*ΔM15Tn10(Tet$^R$)], (Stratagene, Sweden), (a separate colony), was transferred from a plate containing agar-supplemented LB medium with $12.5\,\mu g\,mL^{-1}$ tetracycline to a plate containing 2 mL of LB medium. The culture was incubated overnight with constant aeration at 37°C; 1/10 of overnight culture was transferred to a fresh medium of the same composition and was grown to an exponential phase with aeration at 37°C. When cells reached early log phase ($OD_{660} = 0.5$–$0.6$, corresponding to $7.7 \times 10^8$ cells $mL^{-1}$), the aeration was stopped for 30–40 min in order that the cells restore their F-pili, and the suspension was incubated in a thermostat at 37°C. The concentration of organisms present was checked by standard techniques with the help of light microscopy. Twenty phages per bacterium were used for infection. Upon direct addition of the phages, the culture was incubated at 37°C in a thermostat without shaking, in order for phage particles to sorb at the surface of the pili. After that, the cells were prepared for EO measurements as described earlier.

## 3. Results and discussion

### 3.1 *Electro-optical study of ampicillin effect on* E. coli *cells*

The biological activity of β-lactamic antibiotics is determined to a large measure by their ability to interact with the cell surface and to change the barrier properties

of the cytoplasmic membrane. Because ampicillin is active toward several Gram-negative rods, we used *E. coli* cells for study.

We studied the electrophysical characteristics of strains K-12 (ampicillin-sensitive) and K-12 (pUC-18) (ampicillin-resistant). We also examined the change occurring in the cell-suspension EO parameters during transfection of the ampicillin-sensitive strain XL-1 to an antibiotic-resistant one by using the ampicillin-resistant phage plasmid pHEN1.

We first studied changes occurring in the EO characteristics of a suspension of the ampicillin-sensitive K-12 during ampicillin incubation. To this end, ampicillin was added to a suspension of *E. coli* K-12 cells, previously prepared for EO measurements, to final concentrations at 25, 50, 75, 100, 150, and 250 $\mu g\,mL^{-1}$. Next, the cells were incubated with the antibiotic at 30°C, and the cell-suspension OS were measured. As a result, we established that the OS changes of K-12 cells incubated with various antibiotic concentrations took place at the first five frequencies of the orienting electric field (10–1000 kHz). No substantial changes were noted at higher frequencies. For convenience of data presentation, we used the quantity $\delta OD_{control} - \delta OD_{experiment}$ at an orienting-field frequency of 52 kHz. The greatest decrease in the magnitude of the EO signal occurred at an ampicillin concentration of 50 $\mu g\,mL^{-1}$ (figure 1). The suspension-OS changes did not depend on the incubation time with increasing concentration of the antibiotic.

Depending on the molecular mechanism involved, antibiotics are characterized by bactericidal and bacteriostatic effect. Ampicillin is bactericidal at concentrations that are two- to ten-fold higher than those needed for the bacteriostatic effect to take place [3]. Bacteriostatic effect is produced at low concentrations of $\beta$-lactams; as a result cells lose their ability to form barriers during division, which hinders cell division



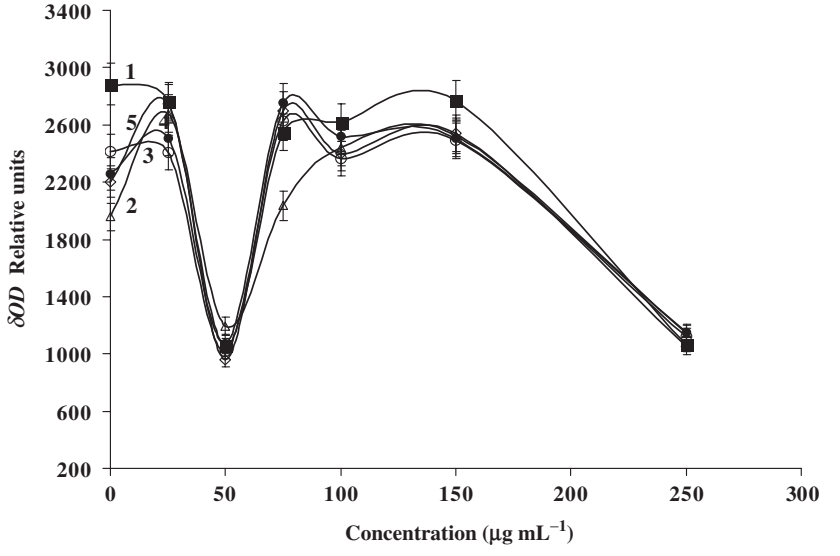


Figure 1. Dependence of the change in the magnitude $\delta OD_{control} - \delta OD_{experiment}$ at an orienting field frequency of 52 kHz in K-12 cells suspended in distilled water (conductivity, 1.8 $S\,m^{-1}$), obtained after incubation with various concentrations of ampicillin for various times. (1) 5 min; (2) 15 min; (3) 30 min; (4) 60 min; (5) 150 min.

and causes the formation of filamentous bacteria. Bactericidal effect is produced with an increase in the antibiotic concentration, and cell lysis occurs at the expense of the fact that at the point of cell division there occurs a deformation of the cell envelope. When high ampicillin concentrations are used, cell lysis occurs before cell filamentousness is expressed. The decrease in the magnitude of the cell EO signal at an ampicillin concentration of $50\,\mu g\,mL^{-1}$ is possibly associated with the cell-envelope deformation occurring at the point of cell division. Thus, at this concentration ampicillin shows bactericidal activity toward *E. coli* K-12. Further work used ampicillin at a concentration of $50\,\mu g\,mL^{-1}$.

We studied the time course of OS changes under the action of ampicillin. To this end, a suspension of K-12 cells was incubated at 30°C for various times (5, 15, 30, 60, and 150 min), prepared for analysis, and used to record the cell-suspension EO parameters. The changes in OS were already evident at 5 min after ampicillin treatment of the cells (figure 2(I)). This fact is possibly due to the antibiotic uptake by the cell wall, since it is known that the rate of antibiotic uptake by the cell is about 2 min [10]. The maximum decrease in the magnitude of the EO signal was observed after 15 min incubation, possibly due to the cell-wall deformation. After a 30 min incubation, there was an increase in the EO signal, in agreement with the data in the literature indicating that maximum ampicillin activity is observed after a 30 min action period [10].

We next studied the electrophysical characteristics of cells of the antibiotic-resistant *E. coli* strain K-12 (pUC-18), having the ampicillin-resistance plasmid pUC-18. To this end, $50\,\mu g\,mL^{-1}$ of ampicillin was added to the suspension, and OS changes were recorded after 5, 15, 30, 60, and 150 min of incubation at 30°C. As a result (figure 2(II)), we found a slight increase in the magnitude of the EO signal after a 5-min incubation with ampicillin. Further incubation did not result in substantial OS changes, which may be considered as a manifestation of resistance of this strain to ampicillin.

The increase in the magnitude of the EO signal after a 5-min incubation is due to the sorption of the antibiotic on the cell, since it is known that cell adsorption is the first stage of microorganism-antibiotic interactions. Ampicillin is adsorbed by both ampicillin-sensitive and resistant bacteria. Adsorption occurs immediately after the antibiotic is added to the suspension, and the ampicillin adsorption process does not depend on the antibiotic concentration in the medium [10].

Thus, there occurred a substantial change in the magnitude of the EO signal after ampicillin incubation of the ampicillin-sensitive strain K-12. No substantial changes in the suspension EO parameters took place after the ampicillin-resistant strain K-12 (pUC-18) was incubated with the antibiotic. Consequently, sensitive and resistant strain of *E. coli* exhibit different dependences of the EO effect on the amount of ampicillin added to the suspension.

To confirm the results, we studied the EO parameters of the ampicillin-sensitive *E. coli* strain XL-1, before and after it was plasmid-modified to become an antibiotic-resistant strain.

For this purpose, XL-1 cells were incubated with ampicillin ($50\,\mu g\,mL^{-1}$) at 30°C for 5, 15, 30, 60, and 150 min. Changes in the EO parameters were then recorded. There was a considerable change in the magnitude of the EO signal irrespective of the incubation time (figure 3(I)). Noteworthy is the fact that the OS-change regularities were close to the results obtained for K-12 cells. Next, we carried out transfection of XL-1 cells by using pHEN1, carrying an ampicillin-resistance gene.

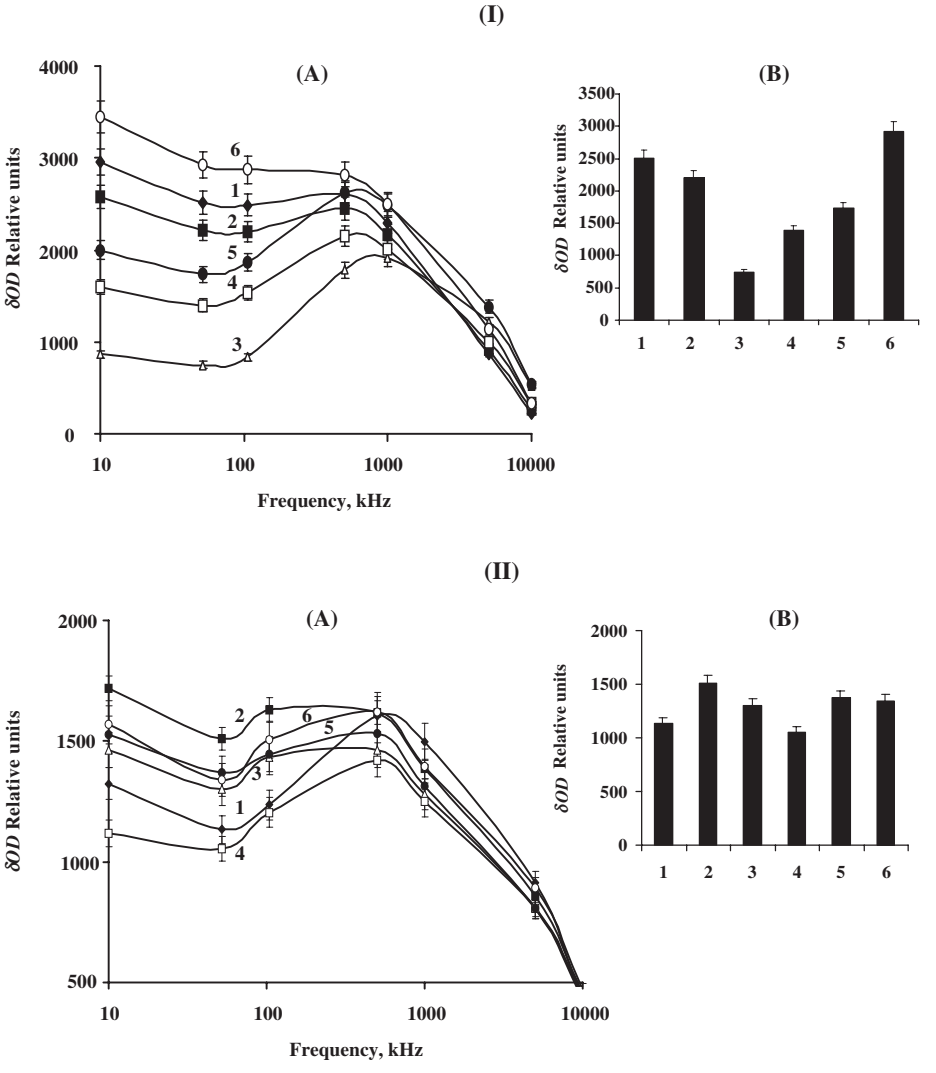


Figure 2. (A) The electro-orientation spectra and (B) relative units at 52 kHz frequency of (I) sensitive strain *E. coli* K-12 and (II) resistance strain *E .coli* K-12 (pUC-18) cells suspended in distilled water (conductivity, 1.8 $S\,m^{-1}$), obtained after incubation with 50 $\mu g\,mL^{-1}$ of ampicillin for different times. (1) control, without ampicillin; (2) 5 min; (3) 15 min; (4) 30 min; (5) 60 min; (6) 150 min.

It is known that cells of this strain carry an F-episome and elaborate F-pili; therefore, they can be phage-infected [8]. Plasmid pHEN1 is constructed on the basis of the genome M13K07 and carries an ampicillin-resistance gene. It can replicate in the way an ordinary plasmid does; and, owing to the presence of an *ori* replicon of phage M13K07, it can assemble into phagmid particles and infect bacterial cells having an F-episome [9]. After transfection, XL-1 cells carrying plasmid pHEN1, were incubated with ampicillin (50 $\mu g\,mL^{-1}$) at 30°C, and OS changes were recorded after 5, 15, 30, 60, and 150 min. As a result (figure 3(II)), we showed a slight increase in the magnitude of the EO signal, which was analogous to that recorded

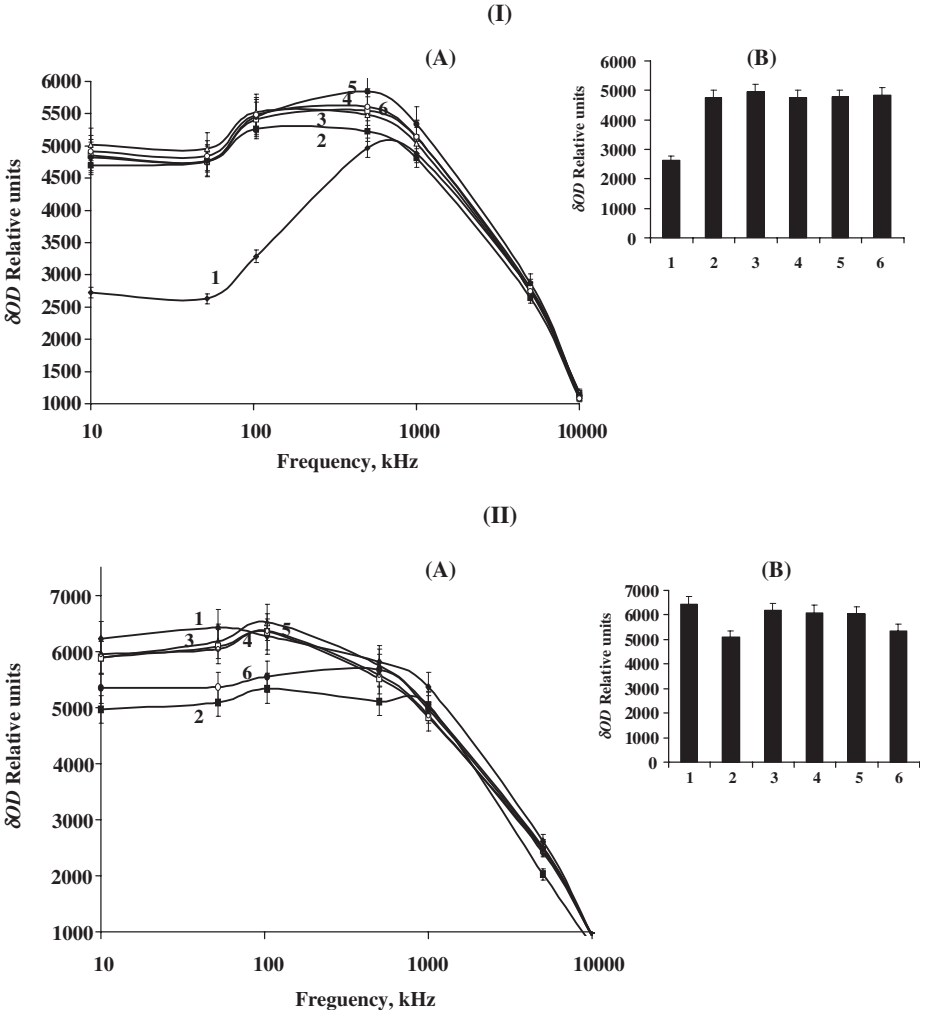


Figure 3. (A) The electro-orientation spectra and (B) relative units at 52 kHz frequency of (I) sensitive strain *E. coli* XL-1 and (II) resistance strain *E. coli* XL-1 (pHEN1) cells suspended in distilled water (conductivity, 1.8 S m$^{-1}$), obtained after incubation with 50 μg mL$^{-1}$ of ampicillin for different times. (1) control, without ampicillin; (2) 5 min; (3) 15 min; (4) 30 min; (5) 60 min; (6) 150 min.

for K-12 (pUC-18). Almost no OS changes were observed on extending the incubation time, attesting that the strain had acquired ampicillin resistance.

Thus, our research shows that when the ampicillin-sensitive strain K-12 and XL-1 are incubated with ampicillin, there occurs a substantial change in the magnitude of the EO signal. In the ampicillin-resistant strains K-12 (pUC-18) and XL-1 (pHEN1), no changes in the suspension EO parameters were found after ampicillin incubation.

### 3.2 *Electro-optical study of kanamycin effect on* E. coli *cells*

An important issue that arises in the development of this method of analysis is the obtainment of an analytical signal with antibiotics having a different mechanism

of action. The action of ampicillin on microbes sensitive to this antibiotic brings about changes in biochemical processes and metabolic pathways, as well as in cell morphology [3]. Therefore, we next studied kanamycin-induced changes in the electrophysical characteristics of the microbial cells. Kanamycin, an inhibitor of protein synthesis, is an aminoglycoside antibiotic of the oligosaccharide family. Once it has entered microbial cells through porin channels, kanamycin suppresses protein synthesis and does not cause changes in cellular morphological characteristics.

Therefore, we next investigated the changes in *E. coli* electro-optic characteristics by using two strains: K-12 (kanamycin-sensitive) and K-12 (PMMB-33) (kanamycin-resistant). We first performed a time-history study of K-12's electro-optic characteristics as affected by kanamycin. To this end, we added the antibiotic (final concentrations, 5 and 10 $\mu g\,mL^{-1}$) to a K-12 suspension. The concentration of 5 and 10 μg mL were chosen because kanamycin is active at these concentrations [3]. The cells were incubated with the antibiotic at 30°C for 5, 15, 30, 60, and 150 min; then, they were prepared for electro-optic analysis and were used in the measurement of orientation spectra. No OS changes were found when a low antibiotic concentration (5 μg mL) was used (figure 4(I)). The maximum decrease in the magnitude of the electro-optic signal occurred at a kanamycin concentration of 10 μg mL (figure 4(II)). The OS changes of the suspensions of K-12 cells incubated with various antibiotic-concentrations occurred at the first five frequencies of the orienting electric field (10–1000 kHz). No substantial changes were seen at higher frequencies. The decrease in the magnitude of the EO signal observed at 10 μg mL may have been due to the bactericidal action of kanamycin on K-12 cells, since it is known that this antibiotic does not cause cell lysis. The mechanism of kanamycin's antimicrobial action is associated with the suppression of protein synthesis, followed by suppression of nucleic acid synthesis and disruption of cell-wall formation. It is known that aminoglycosides find their way through the outer membrane of Gram-negative bacteria via porin channels. Aminoglycosides displace magnesium ions from the outer leaflet of the outer-membrane; the ensuing partial degradation of the membrane leads to enhanced penetration of aminoglycosides [4]. Data from a time-history study of kanamycin (10 μg mL)-induced changes in cellular EO parameters show that the magnitude of the EO signal decreased as early as 5 min after cell treatment with the antibiotic (figure 4(II)). This can be explained by the partial degradation of the cell membrane, allowing entry of the antibiotic into the cell [4]. After 15-min incubation of the cells with kanamycin, there was a further gradual decrease in the magnitude of the EO signal. These changes may have been due to the kanamycin-induced biochemical processes in microbial cells, the suppression of nucleic acid synthesis, and the disruption of cell-wall formation. Thus, the EO-parameters changes of the kanamycin-incubated K-12 cells may indicate the entry of antibiotics into the cells; that is, K-12 is sensitive to kanamycin. In a control treatment, we plated the cells out on kanamycin (5 and 10 μg mL)-containing nutrient medium LB. The cells were incubated at a costant temperature of 30°C for 1 h. At a 5 μg mL kanamycin, the growth of the culture on LB medium was insignificant; at 10 μg mL kanamycin, no culture growth was observed.

Thus, incubation of the sensitive strain (K-12) with 10 μg mL kanamycin caused a substantial change in the magnitude of the EO signal and caused no change with 5 μg mL kanamycin. This was corroborated by the results obtained from cell platings on a kanamycin containing medium.

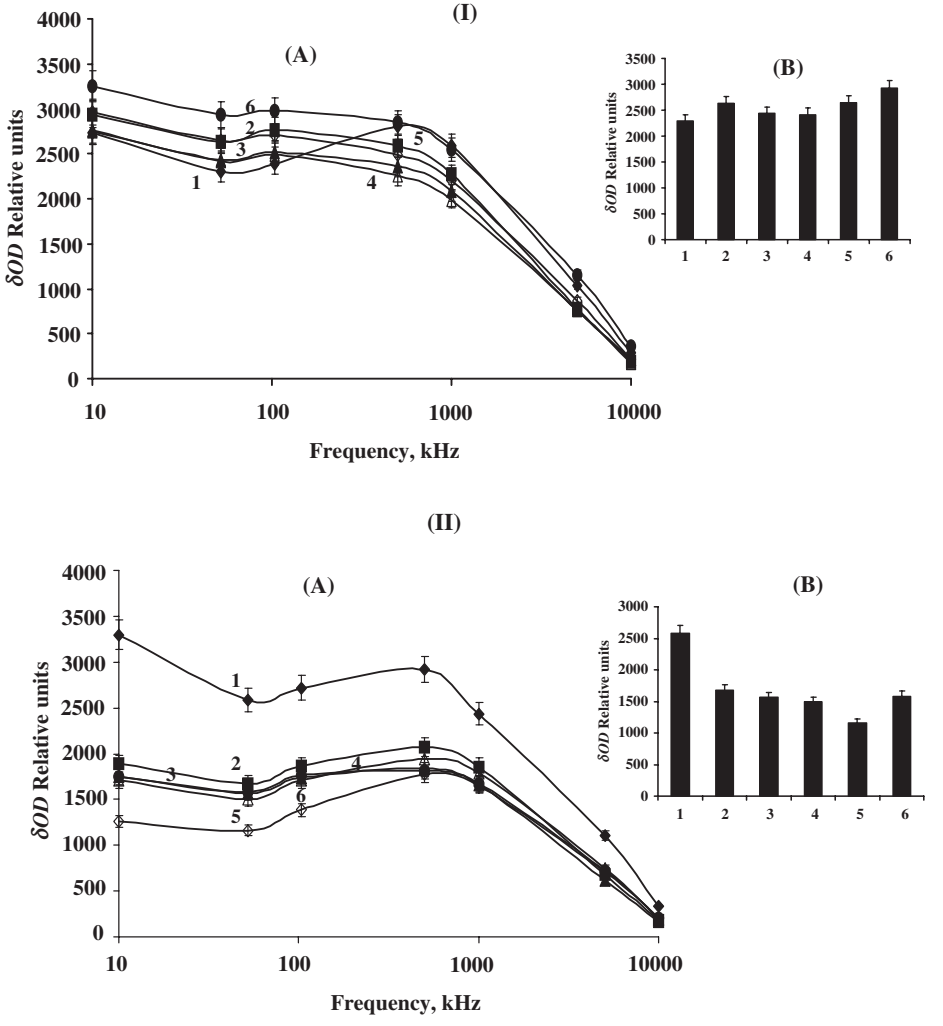


Figure 4. (A) The electro-orientation spectra and (B) relative units at 52 kHz frequency of *E. coli* K-12 cells suspended in distilled water of (conductivity 1.8 S m$^{-1}$), obtained after incubation with 5 μg mL$^{-1}$ (I) and 10 μg mL$^{-1}$ (II) of kanamycin for different time. (1) control (no kanamycin); (2) 5 min; (3) 15 min; (4) 30 min; (5) 60 min; (6) 150 min.

We also ran control experiments to study the EO parameters of the kanamycin-resistant *E. coli* strain K-12 (PMMB-33). To this end, we added kanamycin (10 μg mL) to the cell suspension and measured OS at 30°C at 5, 15, 30, 60, and 150 min after the beginning of the incubation (figure 5). The magnitude of the EO signal at an orienting-field frequency of 52 kHz was used for ease of data presentation (figure 5). No substantial changes in the magnitude of the EO signal were noted after cell incubation with kanamycin (figure 5), which can be considered as a manifestation of resistance of K-12 (PMMB-33) to kanamycin.

Thus, incubation of strain K-12 (kanamycin-sensitive) with kanamycin caused a substantial change in the magnitude of the EO signal. No substantial changes were noted after kanamycin incubation of strain K-12 (PMMB-33) (kanamycin-resistant).

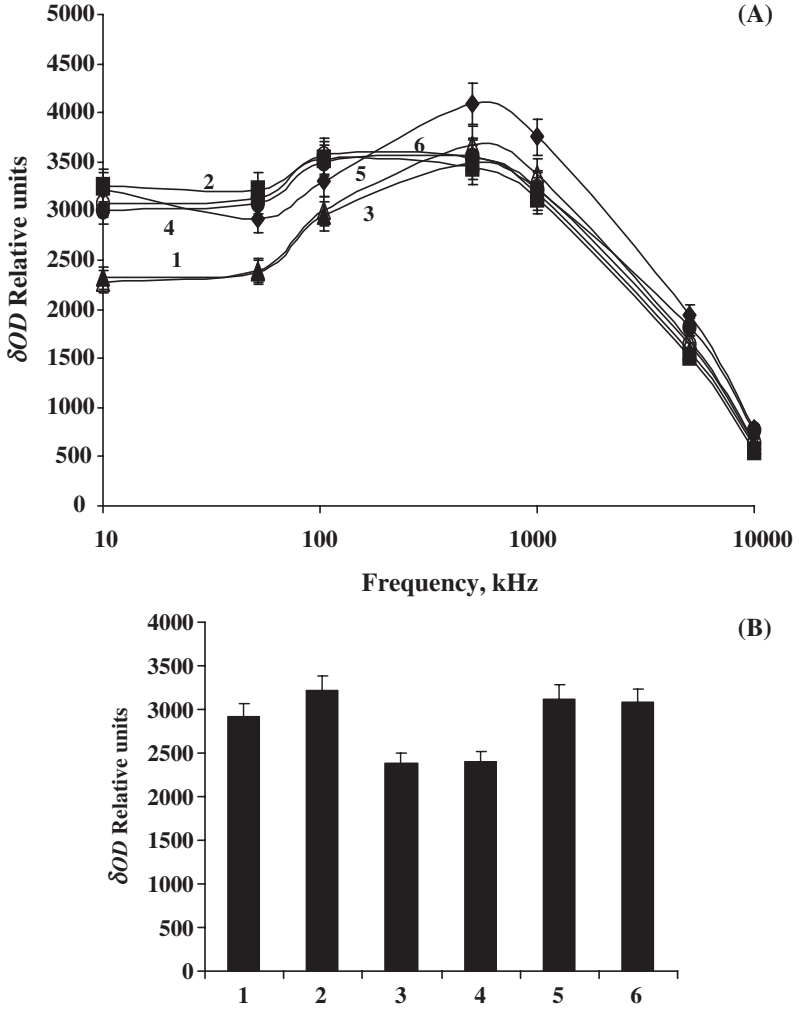


Figure 5. (A) The electro-orientation spectra and (B) relative units at 52 kHz frequency of resistance strain *E. coli* K-12 (PMMB-33) cells suspended in distilled water of (conductivity 1.8 $S\,m^{-1}$), obtained after incubation with 10 $\mu g\,mL^{-1}$ of kanamycin for different time. (1) control (no kanamycin); (2) 5 min; (3) 15 min; (4) 30 min; (5) 60 min; (6) 150 min.

The changes in the electro-optical signal observed under the action of ampicillin or kanamycin may be brought about by osmotic phenomena associated with cell incubation in distilled water. To confirm our results, associated with ampicillin and kanamycin action on the cell-suspension EO parameters, we ran control experiments. To this end, we measured the EO parameters of suspensions of all the strains studied during incubation in distilled water for 5, 15, 30, 60, and 150 min at 30°C without the antibiotic added. No substantial changes in the bacterial-suspension OS were found.

Thus, our research shows that when the ampicillin-sensitive strain K-12 and XL-1 are incubated with ampicillin, there occurs a substantial change in the magnitude of the EO signal. The data obtained in this study show that for the ampicillin- and kanamycin-sensitive strain K-12 incubated with ampicillin and kanamycin, which

have different mechanisms of action, there occur substantial changes in the magnitude of the EO signal. No such changes occur in the ampicillin-resistant K-12 (pUC-18) and XL-1 (pHEN1) or in the kanamycin-resistant strain K-12 (PMMB-33). It follows that the sensitive and the resistant *E. coli* strains exhibit different dependences of the EO effect on the action of ampicillin and kanamycin.

We believe that by recording the OS changes taking place under the effect of ampicillin and kanamycin, we can study the action of the antibiotic on microbial cells and draw conclusions about the existence of resistance to this antibiotic in the cells studied.

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