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Electrooptical Properties of Cells of the Soil Nitrogen-Fixing Bacterium *Azospirillum brasilense*: Effects of Copper Ions

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Abstract—The effects of copper ions on the uptake of some essential metals in the biomass and the electrooptical properties of cell suspensions of the nitrogen-fixing soil bacterium *Azospirillum brasilense* sp. 245 were studied. Copper cations were shown to be effectively taken up by the cell biomass from the culture medium. The addition of copper ions increased the rate of uptake of some other metals present in the culture medium. This was accompanied by changes in the electrooptical characteristics of cell suspension as measured within the orienting electric field frequency range of 10 to 10000 kHz. The effects observed during short-term incubation of *A. brasilense* in the presence of copper cations were less significant than during long-term incubation. These results can be used for the rapid screening of microbial cultures for the enhanced efficiency of sorption and the uptake of metals.

Elucidation of mechanisms of interaction of soil bacteria with metals is an urgent problem of soil microbiology [1]. This is primarily due to the fact that soil microorganisms are in constant and direct contact with various metal-containing components of the soil. The interaction of microorganisms with metals (including toxic metal compounds) plays a substantial role in biogeochemical processes [2].

Copper is an abundant environmental element. It is a soil microelement or a component of various minerals. Copper sulfates, carbonates, phosphates, oxides, and hydroxo salts are the main natural copper-containing minerals. Copper sulfides are formed in underflooded and insufficiently drained soil [3]. In addition, fungicides and herbicides also contribute to contamination of agricultural soils with copper. High concentrations of copper in the soil may cause toxication of plants [4]. The effects of copper ions on the functional activity of biological systems have been recently reviewed in [5]. It is beyond doubt that copper ions have a significant impact on the vital activities of soil microorganisms.

Minute amounts of copper are essential for supporting normal activities of bacteria. Copper is incorporated in certain enzymes (e.g., cytochrome *c* oxidase and oxygenases) [6]. It is presently known which genes control copper transport and homeostasis in *Escherichia coli* in the presence of normal extracellular concentration of the metal [7]. However, if copper concentration in the surrounding medium goes above a certain threshold level, this causes a negative effect on the majority of microorganisms. It was shown in [8] that Cu^{2+} cations induce the release of potassium ions, inorganic phosphate, and other low-molecular-weight metabolites from *Pseudomonas syringae* cells. Copper ions are thought to inflict specific local damage to cell membranes.

Microorganism species differ significantly from each other in the concentration threshold of copper toxicity. For example, photosynthesis and nitrogen fixation in cyanobacteria are inhibited in the presence of copper concentrations of 10^{-10} and 10^{-7} M, respectively [9]. A toxic concentration of copper sulfate for *Azotobacter* and *Aspergillus niger* ranges from 10^{-7} to 10^{-6} M [10]. The mechanism of the bactericidal effect of heavy metals, including copper, was suggested by A.P. Maslyukov *et al*. [10]. According to this mechanism, the concentration of copper ions may decline significantly at the early stages of interaction with bacterial culture as a result of metal binding to: (1) dissolved ions and molecular forms (precipitation, formation of complexes); (2) functional groups of amino acid residues of proteins and other molecular structures of the outer envelopes of bacterial cells; (3) components of periplasmic space where metal ions permeate through hydrophilic pores. Unreacted hydrated copper ions are actively transported through cytoplasmic membrane by special protein transporters. This process has been comprehensively studied in *E. coli* cells [11]. It was shown that Cu^{2+} uptake by *E. coli* cells is coupled to proton release from the cells. This antiport is driven by glucose metabolism. The system of energy-dependent efflux of protons from the *E. coli* cells is highly sensitive to copper. Therefore, as copper concentration in the surrounding medium increases, the rate of Cu^{2+} uptake

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by the cells declines, thereby preventing multiple damage to the cell membrane. Perhaps this explains the fact that copper is less toxic than Hg^{2+} or Ag^{+}, because in contrast to Cu^{2+}, both Hg^{2+} and Ag^{+} permeate through the membrane by an energy-independent mechanism.

Soil bacteria of the genus *Azospirillum* have been extensively studied during the last decade. These bacteria belong to the associative nitrogen-fixing microflora of some plants. The associative nitrogen-fixing microflora was shown to exert a positive effect on plant growth and development [12]. However, the effects of soil metals (copper, in particular) on the viability, metabolism, and efficiency of nitrogen fixation in nitrogen-fixing bacteria have not yet been studied sufficiently or comprehensively. Assimilation of some metals by *A. brasilense* was studied only in a few works [13–15].

The goal of this work was to study comparatively the electrooptical properties of the microbial suspension of *A. brasilense* sp. 245 and content of copper and some other metals in cells grown in copper-containing culture medium.

MATERIALS AND METHODS

Microorganisms. Bacterial culture of *Azospirillum brasilense* (J.J. Tarrand, N.R. Krieg, and J. Döbereiner) from the collection of microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov) was used.

Cultivation conditions. Bacteria were grown in 250-ml Erlenmeyer flasks containing 100 ml each of a synthetic medium. The synthetic medium contained 3.0 g/l K_2HPO_4, 2.0 g/l KH_2PO_4, 0.1 g/l NaCl, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 3.0 g/l NH_4Cl, 0.02 g/l $CaCl_2$, 0.02 g/l $FeSO_4 \cdot 7H_2O$, 0.1 g/l $MnSO_4 \cdot 2H_2O$, 0.002 g/l $Na_2MoO_4 \cdot 2H_2O$, 5.0 g/l malic acid, and 0.1 g/l yeast extract (pH 6.86). Copper was added as $CuSO_4 \cdot 5H_2O$ up to a final concentration of 0.2 mM. The control culture medium was copper-free. Bacterial cultures were incubated on a shaker for 18 h at 32°C. To test the effects of short-term incubation with copper ions, bacterial cells grown in the copper-free medium described above were washed with deionized water, treated with 0.2 mM solution of $CuSO_4 \cdot 5H_2O$, incubated for 1 h, and washed again with deionized water. The electrooptical characteristics of the resulting cell suspension were tested. The yeast extract was from Difco (USA). The salts used in this work were analytical grade products from Serva (USA).

Measurement of orientation spectra of cells. The orientation spectra (OS) of cells were measured using an ELBIC electrooptical analyzer (Institute of Applied Microbiology, Russia) at 670 nm relative to a vacuum. The measured cell volume was 1 ml; the cell concentration (in optical density units), $D_{670} = 0.45–0.50$. Before analysis, bacterial cells were triple washed by centrifugation (5500 *g*, 5 min) and resuspended in a small amount of deionized water. The resulting suspension was centrifuged (1000 *g*, 1 min) to eliminate cell conglomerates, and supernatant was used in further experiments. The resulting cell preparations were incubated for 30 min at 35°C and assayed electrooptically. A set of discrete frequencies of orienting electric field was used (10, 52, 104, 502, 1000, 5020, and 10000 kHz).

The orientation spectrum is the frequency dependence of the difference of optical density values (δD) as measured under conditions of propagation of a unpolarized light beam along and perpendicular to the direction of an orienting electric field vector. This difference was normalized to the optical density level as measured under chaotic cell orientation. It can be assumed that under specific experimental conditions (light beam wavelength, orienting electric field strength, etc.), the OS shape is mainly determined by the frequency dependence of cell polarizability anisotropy [16, 17].

Determination of metal content in the cell biomass. To measure the metal content in the cell biomass, the *A. brasilense* bacteria grown as described above (with or without copper sulfate) were separated from the supernatant by centrifugation, triple washed with 0.85% NaCl solution and bidistilled water, and dried at 50°C for 8 h. Accurately weighed biomass sample (about 3 mg) was mineralized in a Parr Instruments acid bomb (USA) (no. 4745; total volume, 23 ml) in spectroscopical purity grade HNO_3 and deionized water (1 : 1) for 1 h at 110°C. Copper and other essential elements (magnesium, calcium, manganese, and iron) were assayed in the culture medium by flame atomic-absorption spectrometry (acetylene–air flame) using a Perkin-Elmer model 3110 spectrometer (USA). All experiments on metal content measurement in the biomass (including bacterial growth, separation of cells, and spectral analysis of metals) were repeated in triplicate to test the biological reproducibility of the results. The results of parallel measurements of each metal were averaged.

All measurements were performed at 295 ± 3 K unless a different temperature is indicated.

RESULTS AND DISCUSSION

The effects of metals on microorganisms described in the literature were obtained by testing the changes in the biochemical and morphological characteristics of microbial cultures exposed to the metal of interest [18]. However, the problem of rapid and effective detection of the interaction between metals and microbial cells remains unsolved. This problem can be solved either by analytical chemical methods of metal detection in cells and intracellular components or by measuring electrooptical characteristics of the cell biomass. Electrooptical analysis of microbial suspension provides a methodological approach to the problem of measurements of changes in the electrophysical characteristics of microbial cells induced by their interaction with

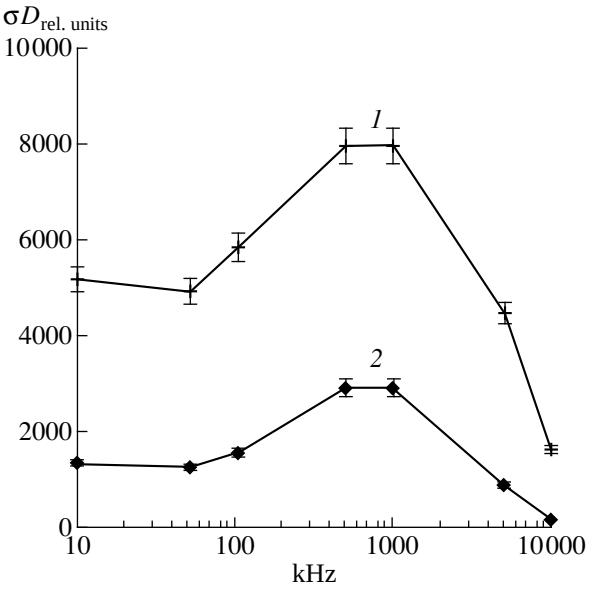

Fig. 1. Electroorientation spectrum of *Azospirillum brasilense* sp. 245 cells grown in the presence of copper ions (0.2 mM) in culture medium: (*1*) control (copper is absent); (*2*) experiment (0.2 mM Cu^{2+}).

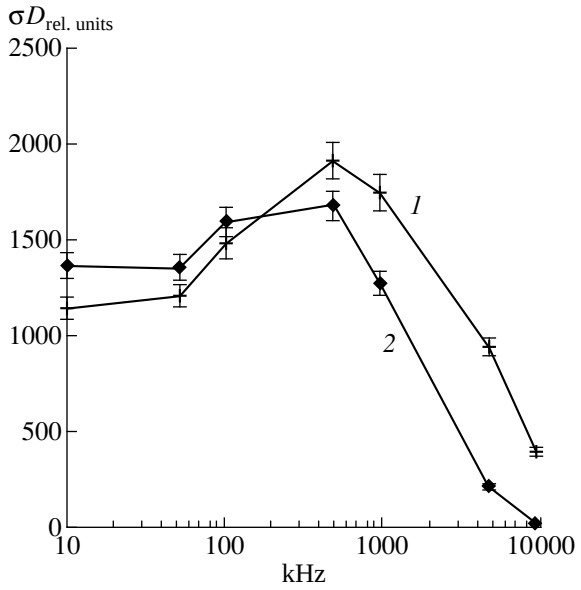

Fig. 2. Electroorientation spectrum of *Azospirillum brasilense* sp. 245 cells under conditions of short-term (1 h) incubation of washed cells in the presence of copper ions (0.2 mM): (*1*) control (copper is absent); (*2*) experiment (0.2 mM Cu^{2+}).

metals. Although similar experiments have been performed earlier [19], a comparative study of electrooptical characteristics of microbial suspensions treated with metals and metal content (accumulation) in cells is of considerable interest, because this provides the possibility to find a direct correlation between these parameters.

The soil bacterium *Azospirillum brasilense* strain sp. 245 was used. Changes in the electrooptical characteristics of microbial cells grown in the presence of copper were studied at the first stage of the study. Bacterial cells were grown in a malate-containing liquid mineral medium in the presence of 0.2 mM solution of $CuSO_4 \cdot 5H_2O$. When bacterial cells were grown, they were washed and placed in an electrooptical cell. It is seen from the results of these experiments (Fig. 1) that the electrooptical properties of the *A. brasilense* sp. 245 cells grown in the presence of copper ions changed throughout the whole frequency range tested. However, the most pronounced effect was observed within the frequency range from 10 to 1000 kHz.

We also studied the uptake of copper and other essential elements (Mg, Ca, Mn, and Fe) by the bacterial cells (table).

We found that copper cations are effectively taken up by the cell biomass from the culture medium. The addition of copper also increased the content of other metals tested.

Changes in the electrooptical characteristics of the *A. brasilense* sp. 245 cells in the presence of copper ions are probably due to partial adsorption of copper ions by cell surface structures and/or changes in the efficiency of assimilation of certain metals by bacterial cells. For example, these changes are manifested in the electrooptical characteristics of both whole cells and cell membranes [15, 20, 21]. It was shown recently [21] that the Cu^{2+} content in cell membranes of bacteria grown in the presence of copper ions was comparable with the Cu^{2+} content averaged over the whole cell biomass. In addition, these cells took up significantly more magnesium than membranes of control bacteria grown in the absence of copper ions in the culture medium [21]. According to Fourier-transform IR spectroscopy, copper ions also modify the phospholipid composition of bacterial membranes [21]. This finding is generally consistent with the effects of ions of heavy metals (including copper) on the lipid composition of membranes of other microorganisms during their adaptation to intoxication with metals [22, 23]. The aggregate of these processes modifies the cell surface properties, thereby changing the electrooptical characteristics of the cells. Because the *Azospirillum* cells in the presence of copper ions accumulated more Mg^{2+} [21], it is interesting to note that magnesium and calcium exert protective effects against the Cu^{2+}-induced inhibition of proton transport in *Saccharomyces cerevisiae* [24].

Thus, bacterial growth in the presence of copper ions is accompanied by Cu^{2+}-induced changes in enzymatic biosynthetic processes in growing cells. In our opinion, the processes induced by short-term exposure of bacterial cells to copper compounds are also of significant interest, because copper adsorption to cell-surface structures is a short-term process. To test these

Metal content in *Azospirillum brasilense* sp. 245 cells

$[Cu^{2+}]$ in medium	Metal content, mg per g dry weight				
	Cu	Mg	Ca	Mn	Fe
Control (copper is absent)	–	1.01 ± 0.42	9.5 ± 3.1	1.34 ± 0.71	4.3 ± 2.9
0.2 mM	2.0 ± 1.3	5.0 ± 2.5	19.0 ± 5.6	3.5 ± 3.1	14.9 ± 10.6

effects, the experimental procedure was slightly modified at the next stage of the study. Bacterial culture was grown in the absence of copper ions, washed with deionized water, treated with 0.2 mM solution of $CuSO_4 \cdot 5H_2O$, incubated for 1 h, and washed again with deionized water. The electrooptical characteristics of the resulting cell suspension were tested as described above (Fig. 2).

Although the electrooptical characteristics of cells in this case also changed, the character of the changes differed from that described above. The electrooptical effect changes in cells subjected to short-term exposure to copper ions were observed at a frequency of 10 kHz and within the frequency range of 502 to 10000 kHz. In addition, the effects observed during short-term exposure of bacterial cells to copper cations were less significant than during long-term incubation in the presence of metal cations. The character of changes in the electrooptical characteristics of the *A. brasilense* sp. 245 cells in our experiments was found to be similar to that described by Ivanov *et al*. [19] in *E. coli* K-12. As in the case of colibacteria, the copper-induced changes in the high-frequency segment of the electrooptical spectrum of *A. brasilense* are characterized by a decrease in the high-frequency maximum and a shift of the high-frequency slope toward a lower frequency. According to Ivanov et al. [19], this can be explained by the different sensitivity of different bacterial species to different damaging (modifying, in general case) physical and chemical factors. These factors deteriorate the barrier function of cytoplasmic membrane and induce the release of potassium ions and other low-molecular-weight components from cells (see also [8]), thereby decreasing the cytoplasm conductance of cells exposed to deionized medium. As a result of these processes, the high-frequency segment of the electrooptical spectrum of cell suspension is changed.

A comparison of Figs. 1 and 2 shows that long-term incubation of the *A. brasilense* sp. 245 culture in the presence of copper ions causes more significant changes in the electrooptical characteristics of cell suspension than short-term exposure to solution of copper ions. This conclusion is consistent with a significant rate of accumulation (assimilation) of copper ions and an enhanced rate of assimilation of a number of essential elements by the *A. brasilense* cells (table). It is obvious that relatively fast adsorption of metal ions to the cell surface is a dominant process under conditions of relatively short-term exposure of cell culture to copper ions. The interaction between bacteria and cations of certain heavy metals (including copper) can be described by rather simple models (e.g, Freundlich's adsorption isotherm) [25].

It should also be noted that adding copper ions to the culture medium containing metal-coordinating agents (phosphate, anions of organic acids) gives rise to the formation of molecular and/or ion forms of copper other than the initial hydrated divalent copper cations.

A certain qualitative difference between the electrooptical characteristics of the *A. brasilense* cells grown in the presence of copper ions, subjected to short-term exposure to pure aqueous solution of Cu^{2+}, and in the control culture (Figs. 1 and 2) can be attributed to the different character (more probably, different result) of interaction between the cell surface and different chemical forms of divalent copper.

Thus, measurements of electrooptical characteristics of microorganisms can be used for monitoring the degree of interaction of microbial cells with copper ions (perhaps other cations, including cations of toxic heavy metals) and for a rapid screening of microbial culture efficiency in various processes associated with sorption of metals by microbial cells.

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