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EXPERIMENTAL ARTICLES

Application of Enzyme Immunoassay for Detection of the Nitrogen-Fixing Bacteria of the Genus *Azospirillum* in Soil Suspensions

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Abstract—The enzyme immunoassay of soil suspensions was performed using antibodies against the lipopolysaccharides of *Azospirillum brasilense* model strains in order to assess the monthly population dynamics of the *A. brasilense* Sp245 introduced into the soil samples in laboratory simulation experiments and to determine the abundance of azospirilla in the various soils of the Saratov oblast. In laboratory simulation experiments (a sample of southern chernozem was used), the maximum amount of the antigen in question was detected in soil suspensions on day 7 of the one-month experiment on the introduction of bacterial cells into soil samples. Analysis of the major soil types of the Saratov oblast (saline, alluvial, and grey forest soils, as well as typical and southern chernozem) revealed the predominance of azospirilla antigens of the Sp245 serotype; significant amounts of the serotype Sp7 antigens were detected in southern chernozem.

Key words: enzyme immunoassay, soil, *Azospirillum*, antibodies, lipopolysaccharides.

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The soil nitrogen-fixing bacteria of the genus *Azospirillum* are a model widely used in studies of microbial–plant interactions [1, 2]. Progress in the study of these bacteria as potential stimulators of plant growth depends significantly on the development of rapid and reliable methods for qualitative and quantitative determination of these microorganisms in soil. The diagnostic systems based on antibodies (Ab) are very convenient tools for monitoring various microorganisms. Enzyme immunoassay (EIA), a technique to reveal antigens (or antibodies), which is based on detection of the antigen–antibody complex by the introduction of an enzyme marker into one of the reaction components, is one of the most efficient serological approaches.

For bacteria of the genus *Azospirillum*, Levanony et al., showed the applicability of EIA for cell identification in pure and mixed cultures, as well as in the plant rhizosphere, using antibodies against whole intact cells of *A. brasilense* Cd [3]. However, the data were subsequently obtained indicating a wide-range specificity of the antibodies against whole *Azospirillum* cells which interact, among others, with the pool of protein antigens (Ag) conservative within the genus [4–6]. It is obvious that, for correct detection of *Azospirillum* strains belonging to certain serological groups, strain- or group-specific antibodies should be used.

The study of the antigenic properties of the cell surfaces of the type and model *Azospirillum* strains revealed that the somatic *Azospirillum* antigen or lipopolysaccharide (LPS) is macroheterogeneous and immunochemically heterogeneous; due to its strain variability, it shows great promise for serological studies [5–9]. The immunochemical specificity of the lipopolysaccharides of the model strains *A. brasilense* Sp7 and Sp245 belonging to different serological types was characterized in detail [7, 8], and the efficiency of antibodies against LPS for determination of specific epitopes in bacterial biofilms and quantitative evaluation of the rhizosphere colonization by azospirilla were demonstrated [6, 10].

The aim of this work was optimization of conditions of the solid-phase enzyme immunoassay with the antibodies against lipopolysaccharides in order to reveal the serologically distinct strains of nitrogen-fixing bacteria of the genus *Azospirillum* in soil suspensions.

MATERIALS AND METHODS

Polyclonal rabbit antibodies against the lipopolysaccharides of *A. brasilense* strains Sp7 and Sp245 were obtained as described in [7]. In our experiments, the following main soil types from the Saratov

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oblast were used: saline soil (Algaiskii raion), alluvial soil (Lysogorskii raion), grey forest soil (Tatishchev raion), typical chernozem (Lysogorskii raion), and southern chernozem (Saratov raion). Soil samples were collected in the middle of August; plant roots were removed from the samples, and the samples were sifted through a 3.0-mm sieve.

In the simulation experiments on the introduction of bacterial cells into soil samples, the strain *A. brasilense* Sp245 [11] and a sample of southern chernozem soil were used. The bacteria were cultivated at 30°C to the late exponential growth phase in a synthetic liquid malate-containing medium [12] supplemented with NH_4Cl (1 g/l). The cells were washed and resuspended in phosphate buffered saline (PBS). The cell suspensions were then transferred into plastic vessels with soil (1×10^9 cells/g soil). The soil free of the introduced bacterial cells was used as a control. The experiment was performed in triplicate. During the experiment, the soil samples in all the vessels were wetted and stirred. After incubation at room temperature and ambient illumination, changes in the numbers of the introduced bacterial cells were compared with those in the control samples. The sampling was carried out after 60 min, 24 h (1 day), as well as after 7, 14, 21, and 28 days following the introduction of the bacterial suspension into the soil samples.

The enzyme-linked immunosorbent assay (ELISA) was performed in 96-well polystyrene plates (Medpolimer, St. Petersburg, Russia). The tested specimens were immobilized by simple adsorption. In order to block the free sites on the polystyrene surfaces, the wells were treated with 100-μl aliquots of a 0.05% polyethylene glycol 20000 (PEG) solution as a ballast. After removal of the blocking solution, 50-μl aliquots of rabbit antibodies obtained against the LPS (primary Ab) dissolved in PBS with 0.02 % Twin-20 and 0.005 PEG (to prevent nonspecific sorption of the antibodies) (solution *1*) were added to each well. The wells were washed three times with 100-μl aliquots of PBS containing 0.02 % Twin-20 (solution *2*) and supplemented with 50-μl aliquots of solution *1* containing peroxidase-labeled goat anti-rabbit antibodies (2 μg/ml) (Sigma, United States). The wells were washed twice with solution *2* and supplemented with 50-ml portions of the substrate for assessment of the peroxidase activity. The substrate consisted of 0.03% *o*-phenylenediamine and 0.02% hydrogen peroxide dissolved in 0.1 M sodium citrate buffer (pH 4.5). To stop the reaction, the wells were treated with 100-μl aliquots of 1 N sulfuric acid solution. The optical density of the tested samples was measured at 490 nm (D_{490}) using an AIF-Ts-01S immunoenzyme analyzer (ZAO ILIP, St. Petersburg, Russia). The results were statistically examined. The confidence intervals were calculated for a significance level of 95%.

In the studies of the abundance of the *Azospirillum* serological groups in various soils of the Saratov oblast, we used the antibodies obtained against the LPS of the strains *A. brasilense* Sp7 and Sp245 representing the different serological types of azospirilla [8]. Weighed portions of the studied soils were used as the control, and the antibodies against the LPS of Sp7 or Sp245 (primary Ab) were replaced with an equal volume of PBS.

RESULTS AND DISCUSSION

To detect the specific bacterial antigen, an indirect enzyme immunoassay was used (a method in which the stained product is visualized due to the interaction of the antigen–antibody complex with anti-immunoglobulin antibodies conjugated with the enzyme). To detect the bacterial antigen, weighed portions (1 g) of soil were thoroughly resuspended in 10 ml of 0.05 M sodium carbonate buffer (pH 9.6). The obtained suspension was agitated manually by vigorous shaking for 30 s; then, 250 μl of the suspension containing 25 mg of soil was diluted with the same buffer to the final volume of 1 ml. The aliquots (50 μl) of successive twofold dilutions of the specimens were introduced into the plate wells for the enzyme immunoassay, and the number of each well corresponded to the degree of dilution. All analyses were performed in duplicates. The antigen-free wells that contained sodium carbonate buffer without the antigen were regarded as zero controls determining the value of optical density deduced from all the values obtained for each specimen.

The experimental evaluation of the population dynamics of the *A. brasilense* Sp245 cells introduced into the soil samples was preceded by a stage of optimization of the conditions of the solid-phase enzyme immunoassay. To determine the optimal incubation time for the suspensions, the plates were agitated on a vibration shaker at room temperature for 30 min to ensure the uniform sorption of the antigen from the solution. In half of the plates, the antigen immobilization was stopped at this stage by removing the suspensions under study by decantation; in the remaining half, immobilization was carried out without agitation at 4°C overnight (18 h).

It was demonstrated that even short-term immobilization of the specimens on the solid phase allowed reliable detection of bacteria. Nevertheless, an increase in the duration of antigen immobilization had a positive effect on the experimental results: the difference in the results of the 30-min and 18-h adsorption was $10 \pm 2\%$ (data not presented). It was demonstrated that the optimal concentration of antibodies against lipopolysaccharides varied 40–180 μg/ml (the concentrations from 40 to 160 μg/ml were compared), since a further increase in the primary antibody's concentration did not increase the sensitivity due to the binding site's saturation in the wells (Fig. 1). The optimal duration of the enzymatic reaction was 4 min, since in this case, the D_{490} values of all the samples studied ranged from 0.2 to 1.0.

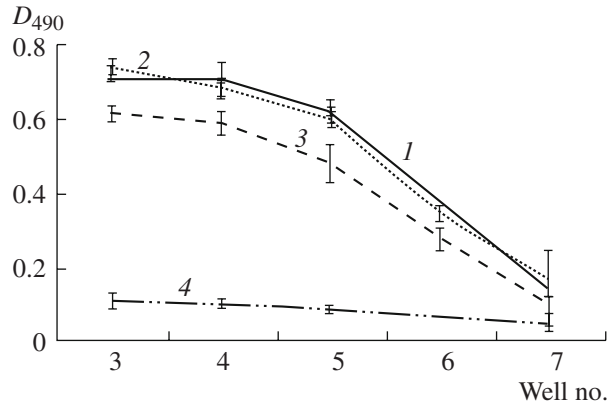

Fig. 1. The results of enzyme immunoassay of the soil samples (southern chernozem) with various concentration of the antibodies against LPS_{Sp245}: 160 μg/ml (*1*), 80 μg/ml (*2*), and 50 μg/ml (*3*). Control (*4*) is the result of the enzyme immunoassay with PBS instead of the primary antibodies. The number (no.) of a well corresponds to the relevant two-fold dilution of the sample.

All the results of the enzyme immunoassay of soil suspensions presented below were obtained in the course of overnight (18 h) adsorption of the specimens in the plate wells, 50 μg/ml of rabbit antibodies, 2 μg/ml of peroxidase-labeled goat anti-rabbit antibodies, and 4 min as the time of the chromophore reaction.

The amount of bacterial lipopolysaccharides in the specimens of inoculated soil at various stages of the experiment was estimated by comparing the D_{490} values of the experimental and control specimens. The statistical analysis of the results consisted of determination of the difference (%) between these values for each dilution: $(D_{490(test)} - D_{490(control)})/D_{490(control)} \times 100\%$. The final result (Fig. 2a) shows the average value (simple average).

It is well known that, in most cases, the dependency of the detected values on the analyte concentration in the course of enzyme immunoassay is more or less nonlinear [13]. The traditional approaches of curve linear-

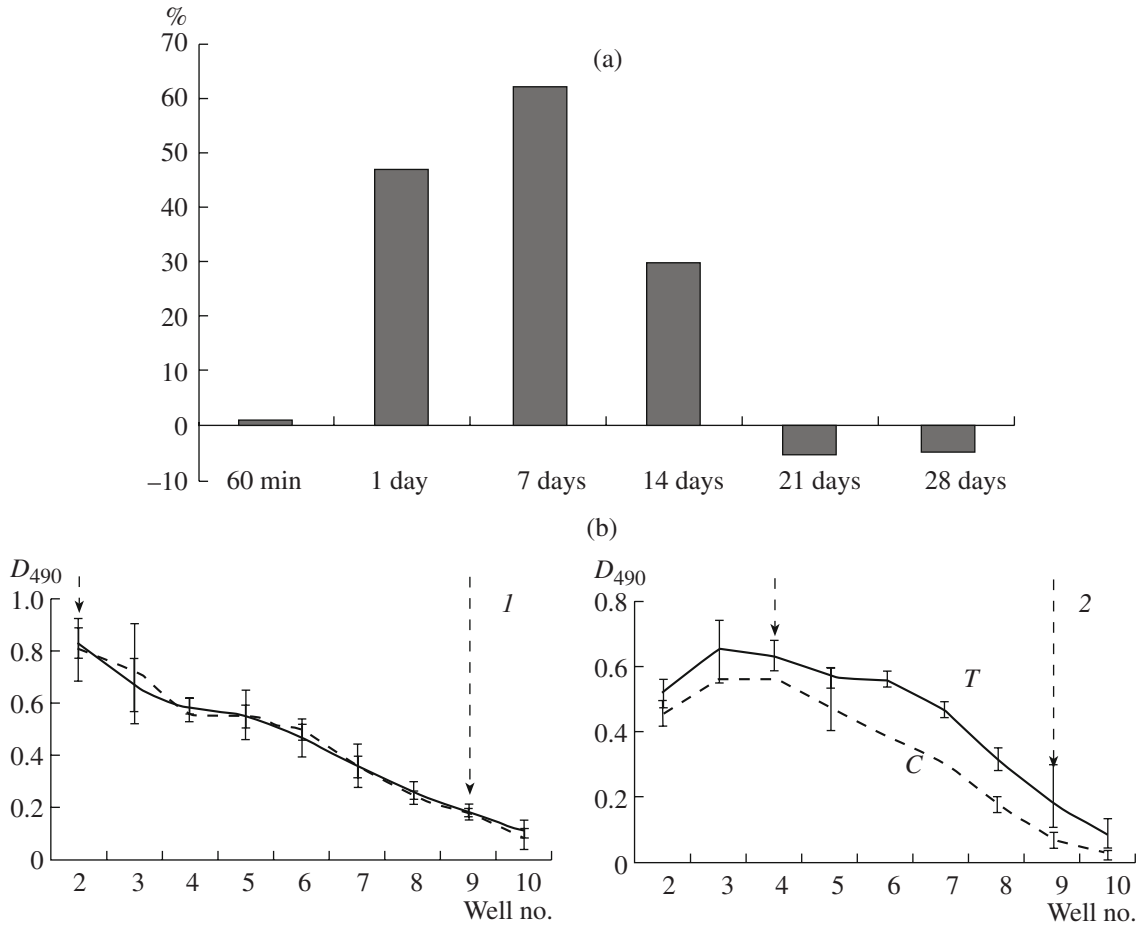

Fig. 2. (a), The results of enzyme immunoassay of the soil samples (southern chernozem) using the antibodies against LPS_{Sp245} after the introduction of the *A. brasilense* Sp245 cell suspension; (b), Differences in the D_{490} values of the experimental and control soil samples in 60 min (*1*) and 7 days (*2*) after the introduction of the *A. brasilense* Sp245 cells (the vertical arrows indicate the ranges of values that were used during the analysis of the results obtained). The number (no.) of a well corresponds to the relevant two-fold dilution of the specimen sample. *C*, control, *T*, experiment.

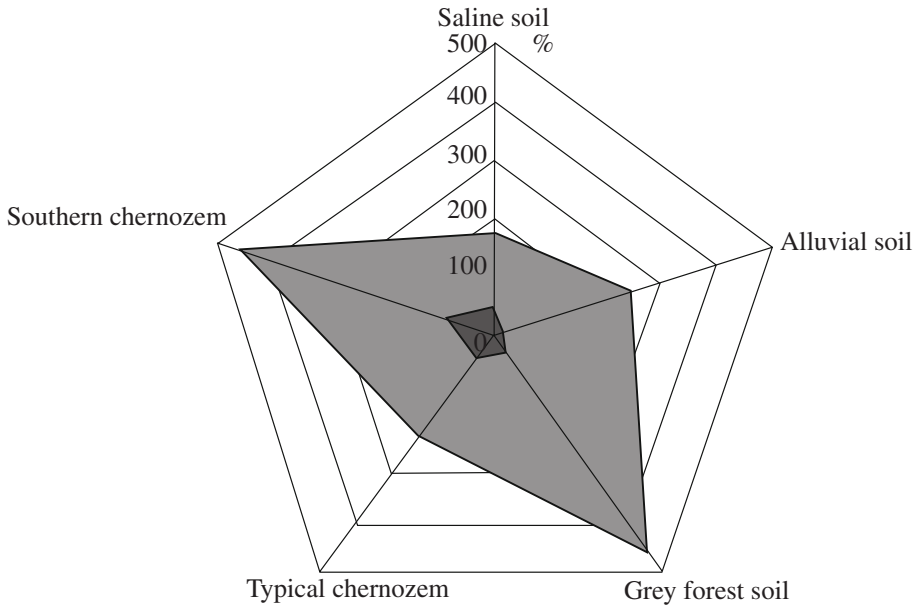

Fig. 3. Comparative enzyme immunoassay of soil samples from the Saratov oblast using the antibodies against LPS_{Sp245} and LPS_{Sp7} (light-gray and dark-grey fields, respectively). For explanation, see text.

ization do not allow us to minimize the so-called "matrix effect" of a specimen [13] represented by a distinctive bend of the curve in the region of high antigen concentrations. Moreover, analysis of such a complex matrix as soil is thwarted by the presence of various interfering factors affecting an enzymatic reaction in each specific case. In this connection, we considered it appropriate to register the difference in the D_{490} values for the test and control specimens in the region of approximate linear dependency of the optical density on the degree of dilution, rather than the absolute D_{490} values.

Figure 2b shows the plots demonstrating the differences between the D_{490} values of the test and control soil samples obtained 60 min (*1*) and 7 days (*2*) after the introduction of the *A. brasilense* Sp245 cells. These plots were used to demonstrate the ranges of the values used during the analysis of the results obtained.

The results of enzyme immunoassay of the soil suspensions allowed us to study the population dynamics of azospirilla in the soil samples inoculated with *A. brasilense* Sp245 cells. Our experiment was based on the data presented in [10] indicating that the detection dynamics of the specific lipopolysaccharide determinants of *A. brasilense* Sp245 (in the work cited, in root homogenates of the inoculated plants) correlated with the increase in the CFU numbers in the test specimens.

Figure 2a shows that the maximum amount of the tested antigen (which was 60% higher than in the control soil sample) was detected in the soil suspensions on day 7 of the one-month experiment. The sharpest increase (by more than 40%) in bacterial numbers (estimated from the lipopolysaccharide content) in soil was observed during the 1st day after inoculation, which corresponds to the logarithmic growth phase of the culture. On day 14, the amount of bacteria decreased significantly; by the end of the 3rd week of the experiment, it was lower than that in the control (uninoculated) soil sample. This decrease may probably be explained by the competitive interactions between the introduced and indigenous bacteria, since the EIA value of the control soil (Fig. 2b) indicates that the soil samples under study contained *Azospirillum* strains exhibiting the serological cross-reactivity with Sp245.

The abundance of members of the *Azospirillum* serological groups in the various soils of the Saratov oblast was studied. Figure 3 shows the results of the comparative enzyme immunoassay of soil samples. It was found that all the studied soils were characterized by the predominance of the serotype Sp245 antigens; in the samples of southern chernozem and gray forest soil, the highest level of these determinants was detected. At the same time, southern chernozem was distinguished by a more significant content of the serotype Sp7 antigens.

The predominance of the antigens of serotype Sp245 in soil is in accordance with the report on the isolation of *A. brasilense* strain SR75 from the wheat rhizosphere in the Saratov oblast [14]. The identity of the structures of the repeating O-specific LPS polysaccharide links of this strain and strain SP245, as well as the homology between the LPS loci in the plasmids of these strains were demonstrated [15]. The strain *A. brasilense* Sp245 is a highly efficient colonizer of wheat roots [11], and detection of a relatively high content of the specific antigen in soil suggests that the *Azospirillum* strains of the Sp245 serotype contribute significantly to the soil fertility of the Saratov oblast. At the same time, as Figure 2b shows, the nonzero

D_{490} value of the control soil sample used in our model experiments had virtually no effect on the evaluation of the population dynamics of the strain *A. brasilense* Sp245 in soil.

Thus, the proposed protocol of solid-phase enzyme immunoassay using antibodies against lipopolysaccharides as a probe allowed us to reveal the antigen targeting soil nitrogen-fixing bacteria of the genus *Azospirillum* directly in soil suspensions. We believe that this approach can be used for both controlling the behavior of bacteria introduced into soil and for assessing the efficiency of bacterial fertilizers and biocomplexes, as well as for evaluation of the effects of various factors on the soil's microbiocenosis.

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