Microbiology, Vol. 70, No. 4, 2001, pp. 436–440. Translated from Mikrobiologiya, Vol. 70, No. 4, 2001, pp. 503–508.

EXPERIMENTAL ARTICLES

Protective Role of the Polysaccharide-containing Capsular Components of *Azospirillum brasilense*

S. A. Konnova, O. S. Brykova, O. A. Sachkova, I. V. Egorenkova, and V. V. Ignatov

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, pr. Entuziastov 13, Saratov, 410015 Russia

Received May 5, 2000; in final form, February 19, 2001

Abstract—The involvement of the carbohydrate components of the *Azospirillum brasilense* Sp245 capsules in bacterial protection from the action of extreme factors was investigated. The survival of encapsulated and non-encapsulated azospirilla exposed to elevated (46–48°C) and below-freezing (–20 and –70°C) temperatures, extreme pH values (2 and 10), and to drying was studied. High-molecular-weight carbohydrate-containing complexes (lipopolysaccharide–protein complex and polysaccharide–lipid complex) were isolated from the capsular material of azospirilla. It was shown that the addition of these complexes to the suspension of decapsulated cells before exposing them to extreme factors enhanced their survival rates by 15 to 51%.

Key words: azospirilla, capsular polysaccharides, extreme conditions, survival.

To be efficient, the use of azospirilla in agriculture calls for the extensive investigation of factors that may adversely affect their survival. Azospirilla are nitrogen-fixing bacteria living in the rhizosphere of various plants. The capsular material of the azospirilla controls the penetration of oxygen into cells and thus protects their oxygen-sensitive nitrogenase system [1]. Many of the recent studies deal with the effect of unfavorable factors, such as saline stress [2], extreme humidity, and nutrient deficiency [3], on the survival of azospirilla in soil and their colonization of roots. It was shown [4] that the flocculating cultures of azospirilla with abundant cell-surface polysaccharides are good inoculants for plants. It was proposed to use the azospirilla encapsulated with alginate gel as potential inoculants for crops [5]. There is indirect evidence that the exopolysaccharides of azospirilla are extensively synthesized in response to drought and protect them from dehydration [6]. The capsular material of azospirilla contains a number of carbohydrate-containing components [7], some of which, such as the high-molecular-weight lipopolysaccharide–protein complex (LPPC) and the polysaccharide–lipid complex (PLC) [8, 9], may be responsible for their protection from unfavorable factors. The role of capsular polysaccharides is commonly investigated using mutants deficient in the synthesis of particular biopolymers. However, the study of such mutants sometimes gives results that are difficult to unambiguously interpret because of multiple mutations [10]. In view of this, the protective role of capsular glycopolymers was proposed to be investigated by decapsulating living bacterial cells. Such an approach allowed Pirog *et al.* [11] to show that the high survival rate of encapsulated *Acinetobacter* cells is due to the protective action of their exopolysaccharides. However, unlike the capsular material of acinetobacters, that of azospirilla has a complex composition and is rich in carbohydrates, which comprise up to 55% of the capsular material at the exponential growth phase [12]. In view of this, to prove the protective role of the polysaccharide complexes of azospirilla, it was decided to isolate and purify these complexes and then add them to the suspension of preliminarily decapsulated bacterial cells.

The aim of the present work was to study the role of high-molecular-weight capsular polysaccharides in the protection of *Azospirillum brasilense* cells from the adverse effect of extreme factors.

MATERIALS AND METHODS

The strain *Azospirillum brasilense* Sp245 used in this work was a generous gift from J. Döbereiner (Brazil). Bacteria were grown in a liquid synthetic medium [8] at 30°C under shaking. Cells were deprived of capsules by incubating them, under continuous stirring, for 7 days in a 10-fold volume of phosphate-buffered saline (PBS) of the following composition (g/l): KH_2PO_4, 0.43; Na_2HPO_4, 1.68; and NaCl, 7.2 (pH 7.2). At 24-h intervals, cells were harvested by centrifugation and transferred to fresh PBS. The supernatant was concentrated tenfold in a vacuum rotary evaporator and analyzed for the presence of polysaccharides by the double-immunodiffusion method with antibodies raised against the glutaraldehyde-treated *Azospirillum* cells [13].

Polysaccharides were stripped from the surface of *Azospirillum* cells by incubating them in a solution con-

0026-2617/01/7004-0436$25.00

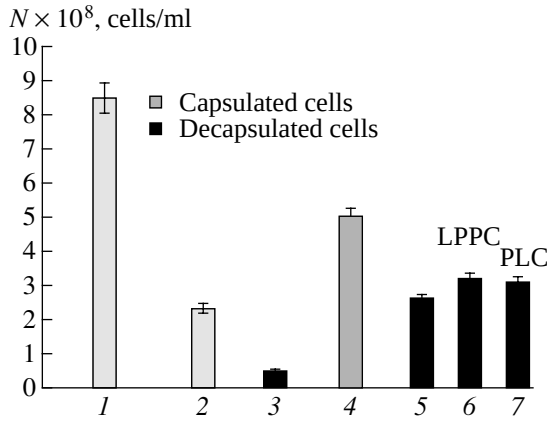

Fig. 1. Effect of the polysaccharide-containing complexes LPPC and PLC on the survival of encapsulated and decapsulated *A. brasilense* Sp245 cells at elevated temperatures. *N* is the concentration of viable cells in the suspension. Bars *1*, *2*, and *4* represent the concentration of viable encapsulated cells before and after heating to 48 and 46°C, respectively. Bar *3* represents the concentration of viable decapsulated cells after heating to 48°C. Bars *5*, *6*, and *7* represent the concentration of viable decapsulated cells after heating to 46°C without and in the presence of LPPC and PLC, respectively.

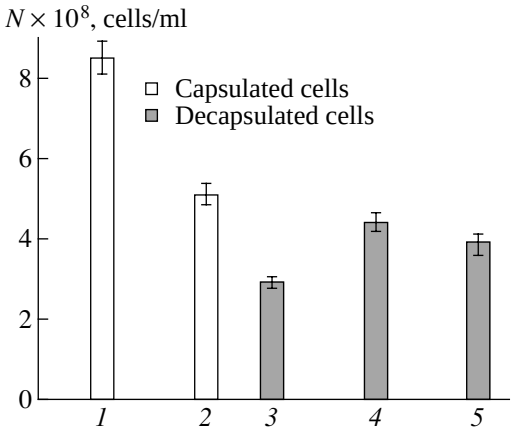

Fig. 2. Effect of the polysaccharide-containing complexes LPPC and PLC on the survival of encapsulated and decapsulated *A. brasilense* Sp245 cells at pH 10. *N* is the concentration of viable cells in the suspension. Bars *1* and *2* represent the concentration of viable encapsulated cells before and after exposure to pH 10, respectively. Bar *3* represents the concentration of viable decapsulated cells before exposure to pH 10. Bars *4* and *5* represent the concentration of viable decapsulated cells after exposure to pH 10 in the presence of LPPC and PLC, respectively.

taining 0.85% NaCl and 0.02% sodium azide under continuous stirring at 4°C for 72 h, after which the cells were removed by centrifugation. The supernatant, which contained capsular polysaccharides, was dialyzed through a 12- to 14-kDa cut-off membrane (Sigma, United States) and then fractionated on a column (55 × 1.8 cm) packed with Sepharose CL-4B (Pharmacia, Sweden) using 0.025 M NH_4HCO_3 (pH 8.1) as the eluant. The fractions eluted from the column were analyzed for carbohydrates by the reaction with phenol and sulfuric acid and for proteins, which were detected by their absorbance measured at 280 nm on an SF-46 spectrophotometer (LOMO, Russia). The fractions that contained high-molecular-weight LPPC and PLC [14] were collected.

The polysaccharide-containing complexes were analyzed for the content of carbohydrates, proteins, nucleic acids, and 2-keto-3-deoxyoctonate (KDO) according to the procedures described elsewhere [14]. Transmission electron microscopy was carried out using a Tesla BS-500 electron microscope at an accelerating voltage of 70 kV. Encapsulated and decapsulated cells were mounted on a Formvar-coated copper or nickel grids and incubated for 20 min in a drop of a phosphate buffer containing 0.2% bovine serum albumin (BSA) and 0.02% Tween-20. Then, the grids were washed with the same buffer from which BSA was omitted, incubated for 30 min in a solution of colloidal gold–labeled wheat germ agglutinin, washed with the buffer and then with bidistilled water, and examined in the electron microscope.

For survival studies, two suspensions of encapsulated and decapsulated cells were equalized in their turbidity (it was adjusted to 1.4 units), which was measured at 420 nm in 1-cm-pathlength cuvettes on a Specol photoelectric colorimeter (Carl Zeiss, Germany). To determine the number of viable cells, 0.1-ml aliquots of bacterial suspensions were diluted 10^6-fold in PBS and plated, in quintuplicate 0.1-ml portions, onto malate-containing nutrient agar. The average number of cells in the suspensions, which was measured in each experiment, was $(8.5 \pm 0.4) \times 10^8$ cells/ml.

The thermotolerance of encapsulated and decapsulated cells was evaluated by measuring their survival after heating at 46 and 48°C for 15 min.

To study bacterial tolerance to freezing, the suspensions of encapsulated and decapsulated cells were kept at –20°C for 2 days and then at –70°C for 5 days.

The effect of pH on bacterial survival was studied by incubating cells in PBS with pH 2 and 10 for 2 h.

To study the resistance of bacterial cells to drying, 2.0-ml aliquots of the two bacterial suspensions were placed in sterile petri dishes and allowed to completely dry at 25°C in a thermostated chamber. After 7 days, 2 ml of sterile distilled water was added to each dish and the number of viable bacteria in the resultant suspensions was determined as described above.

The protective role of the polysaccharide-containing complexes was studied by evaluating bacterial survival in the presence of LPPC and PLSC, which have been added, at a concentration of 0.2 mg/ml, to bacterial suspensions before they were exposed to one of the extreme factors. The working concentration of the complexes was chosen in preliminary experiments, taking into account their yield in the process of isolation

Effect of the polysaccharide-containing complexes LPPC and PLC on the survival of encapsulated and decapsulated *A. brasilense* Sp245 cells upon drying and freezing

Unfavorable factor	Number of viable cells in 1 ml						
	Encapsulated cells	Decapsulated cells	*P**	Decapsulated cells + LPPC	*P***	Decapsulated cells + PLC	*P***
Freezing	$(5.0 \pm 0.1) \times \times 10^4$ [27]	$(9.1 \pm 0.3) \times \times 10^3$ [37]	<0.01	$(1.2 \pm 0.1) \times \times 10^4$ [35]	<0.05	$(1.3 \pm 0.04) \times \times 10^4$ [38]	<0.05
Drying	$(4.5 \pm 0.4) \times \times 10^5$ [33]	$(1.1 \pm 0.3) \times \times 10^5$ [29]	<0.01	$(1.3 \pm 0.1) \times \times 10^5$ [30]	>0.05	$(1.4 \pm 0.2) \times \times 10^5$ [27]	<0.05

Note: Bracketed is the number of replicate experiments. Data are presented for a confidence level of 95%. *P* is the significance level with respect to * encapsulated and ** decapsulated cells.

from the capsular material and the data available in the literature concerning the strain Cd [8].

All experiments were performed in 12–15 replicates (each replicate experiment included 3–5 identical agar plates). The results were statistically processed by the methods described by Rokitskii [15].

RESULTS AND DISCUSSION

The role of carbohydrate-containing capsular complexes in the bacterial tolerance to extreme factors was studied using *A. brasilense* Sp245 cells grown in a malate-containing liquid medium to the late exponential phase (20 h of growth). The cells were deprived of capsules by incubating them in PBS as described in the *Materials and Methods* section. The completeness of the capsule removal from the cells was controlled by the double-immunodiffusion method and transmission electron microscopy. Analysis by immunodiffusion with the antibodies raised against glutaraldehyde-treated azospirilla showed that capsular polysaccharides could not be detected in the PBS as soon as after 6–7 days of incubation, even if concentrated solutions were used for the analysis. It should be noted that these antibodies are, in fact, monospecific to a lipopolysaccharide of the outer membrane of bacteria [13], which is also present in the capsular material of azospirilla.

Earlier, we showed that the surface of washed azospirilla does not interact with the wheat germ agglutinin–colloidal gold complex [9]. Since WGA is capable of interacting with the bacterial capsular material [16], the absence of the interaction between the gold-labeled lectin and the bacterium, which was shown by electron microscopy, implied the complete removal of capsular polysaccharides from the bacterial surface.

The decapsulating incubation of bacterial cells for 7 days led to the death of about 70% of cells originally present in the suspension. In view of this, the suspension of decapsulated cells was concentrated to the initial optical density (OD_{420} = 1.4). The viable cells present in this suspension were motile and could restore their capsules after 2–3 days of incubation.

To elucidate the tolerance of encapsulated and the tolerance of decapsulated *Azospirillum* cells in the presence and absence of polysaccharide-containing complexes, the cells were exposed to extreme temperatures and pH values and to desiccation, i.e., to the extreme factors that many microorganisms have to face in nature.

Temperature is one of the environmental factors that are vital to microorganisms at different developmental stages. Two temperature ranges are of interest from a practical standpoint: the temperature range within which microorganisms can grow and reproduce and extreme temperatures that induce lethal damage to the cell [17]. Azospirilla are mesophilic bacteria: they can grow at temperatures from 4 to 45°C, with an optimum growth temperature of 32–35°C, and the most favorable temperature for nitrogen fixation of 33–40°C [18]. Experiments performed in the present study showed that the number of encapsulated cells that survived exposure to 48°C for 15 min was$(2.3 \pm 0.9) \times 10^8$cells/ml, that is 27% of the initial number of cells (Fig. 1). Decapsulated cells showed a still lower survival rate (6% or $(0.5 - 0.1) \times 10^8$ cells/ml). The same tendency was observed when the exposure temperature was lowered to 46°C: the survival rates of encapsulated and decapsulated cells were 58 and 31%, respectively (Fig. 1). It should be noted that data in the literature on the thermostability of azospirilla are scarce and, to the best of our knowledge, there is no information on the mechanisms providing for their survival in the plant rhizosphere at elevated temperatures.

Bearing in mind that carbohydrates comprise a large portion of the capsular material of azospirilla, especially at the early stages of culture growth [12], and the involvement of exopolysaccharides in the protection of *Acinetobacter* Sp. from damaging factors [11], we suggested that the LPPC and PLC of the capsular material of azospirilla may perform protective functions. Procedures for the isolation of LPPC and PLC from the cell surface were elaborated in our previous works [8, 14]. The extracts purified by gel filtration on a Sepharose CL-4B column represented homogeneous biopolymers. Their analysis confirmed our previous data [14] that LPPC, whose yield is 2.5% of the dry weight of

cells, contains about 22% proteins, 25% carbohydrates, 1.0% nucleic acids, and 1.5% 2-keto-3-deoxyoctonate (KDO) (the presence of KDO indicates that the complex contains a lipopolysaccharide). Correspondingly, PLC contains 60% carbohydrates, 8% protein, 1.2% nucleic acids, and KDO in trace amounts. As shown earlier [14], the major constituents of the LPPC and PLC lipids are hexadecanoic (16 : 0), octadecenoic (18 : 1), and hexadecenoic (16 : 1) acids. This finding is of particular interest, since it is known that glycolipids are responsible for the thermostability of bacterial membranes and that the increase in the content of long-chain saturated and unbranched fatty acids in membrane lipids is one of the protective responses of the thermophilic bacteria to elevated temperatures [19]. The addition of LPPC and PLC at a concentration of 0.2 mg/ml to the suspension of decapsulated cells before heating increased their survival rate by 18 and 16%, respectively (Fig. 1).

After freezing at temperatures typical of the cryopreservation of microbial cultures, the number of viable encapsulated cells was $(5.0 \pm 0.1) \times 10^4$ cells/ml, i.e., 82% greater than that of viable decapsulated cells (see table). The preliminary addition of LPPC and PLC to the suspension of decapsulated cells augmented the number of cells that survived freezing by 25 and 30%, respectively. One of the causes of the adverse effect of below-freezing temperatures on living organisms is their damage at the stages of freezing and thawing. The crystallization of inter- and intracellular water adversely affects biological systems. High-molecular-weight polysaccharide complexes occurring on the cell surface may enhance the cryotolerance of cells by binding water and increasing the viscosity of the intercellular matrix. On the other hand, the unsaturated fatty acids of these complexes may enhance the cryotolerance of bacteria by increasing the flexibility of their plasma membranes and changing their phase transition temperature and permeability [20, 21].

The viability of soil bacteria is largely determined by the concentration of hydrogen ions in the soil solution. *Azospirillum* show the best growth at neutral pH values (6.9–7.8); however, at pH about 5.6 and 8.7, this bacterium remains active [18].

To study the sensitivity of encapsulated and decapsulated azospirilla to the concentration of hydrogen ions, they were placed in media with pH 10 and 2. Incubation at pH 10 led to the death of 40% encapsulated and 66% decapsulated cells (Fig. 2). The addition of LPPC and PLC to the suspension of decapsulated cells raised the number of survived cells by 51 and 35%, respectively. The difference is statistically significant for $P < 0.05$.

Azospirilla turned out to be more susceptible to acidic than to alkaline pH values. The number of the encapsulated cells that survived exposure to pH 2 drastically (by several thousand times) decreased, amounting to $(2.0 \pm 0.3) \times 10^4$ viable cells/ml. Decapsulated cells showed survival rates six times worse, amounting to $(3.6 \pm 0.2) \times 10^3$ viable cells/ml (Fig. 3). The addition of LPPC and PLC to the suspension of decapsulated cells augmented the number of survivors to $(4.8 \pm 0.4) \times 10^3$ and $(4.1 \pm 0.1) \times 10^3$ cells/ml, respectively. Thus, in the presence of the polysaccharide complexes in the suspension, the number of surviving cells showed a statistically significant increase ($P < 0.05$).

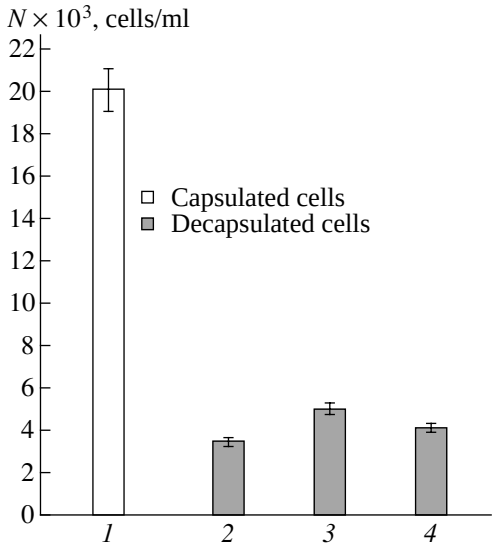

Fig. 3. Effect of the polysaccharide-containing complexes LPPC and PLC on the survival of encapsulated and decapsulated *A. brasilense* Sp245 cells at pH 2. *N* is the concentration of viable cells in the suspension. Bars *1* and *2* represent the concentration of viable encapsulated cells before and after exposure to pH 2, respectively. Bars *3* and *4* represent the concentration of viable decapsulated cells after exposure to pH 2 in the presence of LPPC and PLC, respectively.

Drying is a common phenomenon to rhizosphere microorganisms. In our experiments, the drying of the suspension of capsulated azospirilla reduced the number of viable cells by more than 2000 times. The survival rate of decapsulated bacterial cells exposed to drying was 76% lower than that of encapsulated cells (see table). The addition of PLC and LPPC augmented bacterial survival by 15–21%. However, the statistical processing of these results showed that the difference in the amount of viable cells in the presence and absence of LPPC was statistically insignificant. At the same time, the enhancement of bacterial survival by PLC upon drying was found to be statistically significant.

It can be seen that not only decapsulated but also encapsulated azospirilla are very susceptible to drying, freezing, and acidic pH values. This can be explained by the fact that bacterial cells for the experiments described here were taken from the exponential growth phase, although it is known that the adaptation of azospirilla to extreme environmental conditions involves the production of cysts [3] with the intense

synthesis of exopolysaccharides [6], which occur under unfavorable growth conditions.

To conclude, the encapsulation of the exponential-phase azospirilla makes them more tolerant to the action of some unfavorable factors. This is due, in part, to the protective effect of the complex biopolymers LPPC and PLC, which are located on the cell surface or may release into the medium. The experiments described here made it possible to evaluate the protective effect of the native capsule of azospirilla and its polysaccharide-containing constituents. It is evident that for the inoculation of plants, one should use *Azospirillum* cultures grown under the conditions that are beneficial for the formation of capsules, which promote the survival of azospirilla in soil and their attachment to the plant roots [9].

ACKNOWLEDGMENTS

We are grateful to L.Yu. Matora and O.B. Serebrennikova for the immunodiffusion analysis.

REFERENCES

1. Berg, R.H., Tyler, M.E., Novick, N.J., Vasil, V., and Vasil, I.K., Biology of *Azospirillum*–Sugarcane Association: Enhancement of Nitrogenase Activity, *Appl. Environ. Microbiol.*, 1980, vol. 39, pp. 642–649.
2. Jofre, E., Rivarola, V., Balegno, H., and Mori, G., Differential Gene Expression in *Azospirillum brasilense* Cd Under Saline Stress, *Can. J. Microbiol.*, 1998, vol. 44, no. 10, pp. 929–936.
3. Lamm, R.B. and Neyra, C.A., Characterization and Cyst Production of Azospirilla Isolated from Selected Grasses Growing in New Jersey and New York, *Can. J. Microbiol.*, 1981, vol. 27, pp. 1320–1325.
4. Neyra, C.A., Atkinson, A., and Olubayi, O., Coaggregation of *Azospirillum* with Other Bacteria: Basis for Functional Diversity, *Azospirillum VI and Related Microorganisms*, *Fendrik*, I. *et al.*, Eds., Berlin: Springer, 1995, vol. 37, pp. 429–439.
5. Fages, J., An Optimized Process for Manufacturing an *Azospirillum* Inoculant for Crops, *Appl. Microbiol. Biotechnol.*, 1990, vol. 32, pp. 473–478.
6. Sadasivan, L. and Neyra, C.A., Flocculation in *Azospirillum brasilense* and *Azospirillum lipoferum*: Exopolysaccharides and Cyst Formation, *J. Bacteriol.*, 1985, vol. 163, pp. 716–723.
7. Skvortsov, I.M. and Ignatov, V.V., Extracellular Polysaccharides and Polysaccharide-containing Biopolymers from *Azospirillum* Species: Properties and the Possible Role in Interaction with Plant Roots, *FEMS Microbiol. Lett.*, 1998, vol. 165, pp. 223–229.
8. Konnova, S.A., Makarov, O.E., Skvortsov, I.M., and Ignatov, V.V., Isolation, Fractionation, and Some Properties of Polysaccharides Produced in a Bound Form by *Azospirillum brasilense* and Their Possible Involvement in *Azospirillum*– Wheat Root Interactions, *FEMS Microbiol. Lett.*, 1994, vol. 118, no. 2, pp. 93– 99.
9. Egorenkova, I.V., Konnova, S.A., Fedonenko, Yu.P., Dykman, L.A., and Ignatov, V.V., The Role of the Polysaccharide-containing Capsular Components of *Azospirillum brasilense* in Bacterial Adsorption on Wheat Seedling Roots, *Mikrobiologiya*, 2001, vol. 70, no. 1, pp. 45–50.
10. Vande Broek and Vanderleyden, J., Genetics of the *Azospirillum*–Plant Root Association, *Crit. Rev. Plant Sci.*, 1995, vol. 14, no. 5, pp. 445–466.
11. Pirog, T.P., Grinberg, T.A., and Malashenko, Yu.R., Protective Function of the Polysaccharide Synthesized by *Acinetobacter* sp., *Mikrobiologiya*, 1997, vol. 66, no. 3, pp. 335–340.
12. Dufrne, Y.F. and Rouxhet, P.G., Surface Composition, Surface Properties, and Adhesiveness of *Azospirillum brasilense*: Variation during Growth, *Can. J. Microbiol.*, 1996, vol. 42, pp. 548–556.
13. Matora, L., Solovova, G., Serebrennikova, O., Selivanov, N., and Shchyogolev, S., Immunological Properties of *Azospirillum* Cell Surface: The Structure of Carbohydrate Antigens and Evaluation of Their Involvement in Bacteria–Plant Contact Interactions, *Azospirillum VI and Related Microorganisms*, Fendrik, I. *et al.*, Eds., Berlin: Springer, 1995, vol. 37, pp. 377–382.
14. Konnova, S.A., Makarov, O.E., Skvortsov, I.M., and Ignatov, V.V., The Exopolysaccharides of *Azospirillum brasilense* Sp245 and Sp107, *Mikrobiol. Zh.* (Kiev), 1992, vol. 54, no. 2, pp. 31–42.
15. Rokitskii, P.F., *Biologicheskaya statistika* (Biostatistics), Minsk: Vysheishaya Shkola, 1973, p. 320.
16. Yagoda-Shagam, J., Barton, L.L., Reed, W.P., and Chiovetti, R., Fluorescein Isothiocyanate–labeled Lectin Analysis of the Surface of the Nitrogen-fixing Bacterium *Azospirillum brasilense* by Flow Cytometry, *Appl. Environ. Microbiol.*, 1988, vol. 54, no. 7, pp. 1831–1837.
17. Munblit, V.Ya., Tal'roze, V.L., and Trofimov, V.I., *Termoinaktivatsiya mikroorganizmov* (Thermoinactivation of Microorganisms), Moscow: Nauka, 1985, p. 248.
18. Stephan, M.P., Pedrosa, F.O., and Dbereiner, J., *Physiological Studies with Azospirillum* spp. Associative N_2-Fixation, *Florida*: Boca Raton, 1981, pp. 8–12.
19. Kushner, D.J., Life of Microorganisms at High Salt and Solute Concentrations, *Microbial Life in Extreme Environments*, Kushner, D.J., Ed., London: Academic, 1978. Translated under the title *Zhizn' mikrobov v ekstremal'nykh usloviyakh*, Moscow: Mir, 1981.
20. *Aktual'nye problemy kriobiologii* (Topical Problems in Cryobiology), Pushkar', N.S. and Belous, A.M., Eds., Kiev: Naukova Dumka, 1981.
21. Vorob'ev, V.A., *Simbioticheskaya azotfiksatsiya i temperatura* (The Effect of Temperature on Symbiotic Nitrogen Fixation), Novosibirsk: Nauka, 1998.