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EXPERIMENTAL ARTICLES

Extracellular Protease as a Reversible Adhesion Regulator in *Pseudomonas fluorescens*

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Abstract—The investigation of growth dynamics and protein content in a batch *Pseudomonas fluorescens* culture grown in a synthetic medium with glucose as the sole source of carbon and energy showed that cells reversibly adhere to the walls of the cultivation flask during the first 2–3 h of growth. Over this time period, the total protein content of free and bound cells increased exponentially at a rate of 0.25 h^{-1}, the fraction of proteins in cells being almost the same (60–70%). The protein content in the medium increased from 3 to 50 mg/l, reaching about 30% of the total protein of the culture. The addition of the exponential culture liquid filtrate to the medium together with the inoculum led to the complete inhibition of cell adhesion and a drastic activation of proteolysis, with a concurrent release of more than 80% of cellular proteins into the medium. After 3–5 h of growth, the concentration of extracellular proteins decreased to the control level. Exogenously added proteinase K inhibited cell adhesion, the effect being more pronounced for R-type than for S-type cells. The hypothesis is discussed that the short-term reversible adhesion of cells is regulated with the involvement of a mixture of hydrocarbons, which inactivate the functional activity of bacterial adhesins, and proteases, which digest these adhesins.

Key words: *Pseudomonas fluorescens*, reversible adhesion, regulation, proteinase.

Pseudomonas fluorescens cells are capable of reversible adhesion [1], which is regulated by extracellular antiadhesins (AAs) [1–4]. The mathematical simulation of the adhesion of *P. fluorescens* cells, presented in the accompanying paper [4], showed that there must be at least two types of antiadhesins, one of which (AA_1) blocks the adhesion of free cells, whereas the other (AA_2) causes the desorption of cells attached to a surface. The results of the simulation were confirmed experimentally. In particular, we succeeded in the identification of unbranched *n*-alkanes that served as antiadhesins of the first type [3].

The present work is devoted to the study of antiadhesins of the second type, AA_2. When investigating the mechanism of action of AA_2, it should be taken into account that adhesins are polymeric compounds with two sticky ends, which are responsible for the reversible adhesion of cells [1, 2]. The adhesins of *P. fluorescens* contain a polysaccharide [5–7] and protein components [1, 2], indicating that they, like the adhesins of other bacteria, are glycoproteins [7]. The simplest supposition as to the nature of AA_2 is that it is a hydrolytic enzyme or an enzyme complex degrading adhesins.

The R-type variants of pseudomonads, forming rough colonies [12], are more prone to reversible adhesion than S variants. The adhesion is easily induced by transferring bacterial cells to fresh medium, when the cells primarily respond to the "stress of new conditions" by adhering to the walls of the cultivation flask. This process requires adhesins [1]. The adhesion of cells in a culture can be prevented by adding the filtrate of another culture of the same species together with the inoculum. In this case, the cells seem to receive a signal that the medium is favorable for their growth, and adhesins are either blocked or destroyed by hydrolases. The dynamics and the degree of cell adhesion can be specified either by using R- or S-type cell variants, by transferring cells to a fresh medium, or by adding the culture liquid filtrate to a growing culture. The attachment and detachment of cells must be accompanied by changes in the content of proteins in the medium. These ideas were used as basic when designing experiments to verify the above hypothesis on the nature of the AA_2 antiadhesins of *P. fluorescens*.

In the present paper, we report experimental data on (1) the dynamics of proteins in the medium and in the *P. fluorescens* cells grown in a batch mode in fresh nutrient media and in the same media with the added culture liquid filtrate and (2) the effect of the exogenously added proteinase K on the adhesive properties of pseudomonads.

MATERIALS AND METHODS

Experiments were carried out with the strain *Pseudomonas fluorescens* NCIMB 9046 obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) at the University of Aberdeen (United Kingdom). The strain was grown in M9 medium [8] containing (g/l): Na_2HPO_4, 6.8; KH_2PO_4, 3; NaCl, 0.5;

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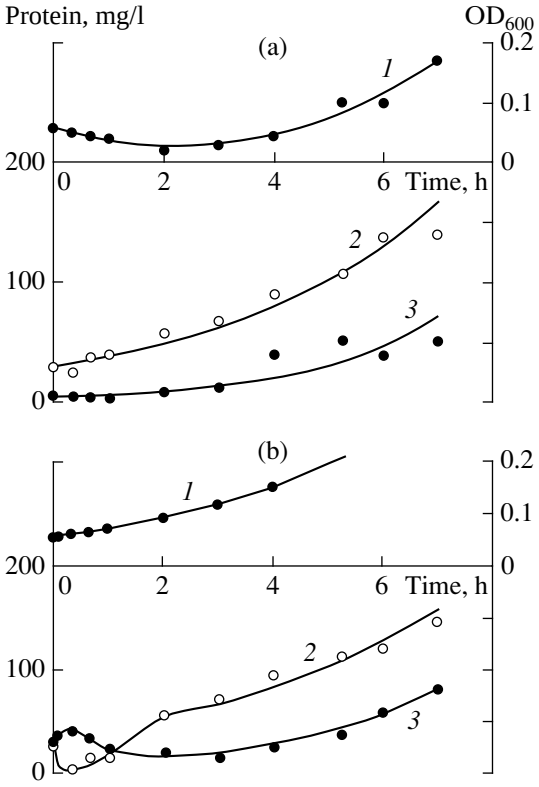

Fig. 1. Growth dynamics of *P. fluorescens* in (a) fresh glucose-mineral M9 medium and (b) the same medium with the added culture liquid filtrate: (*1*) optical density of the culture (is proportional to the number of free cells in the medium); (*2*) cellular protein; and (*3*) extracellular protein.

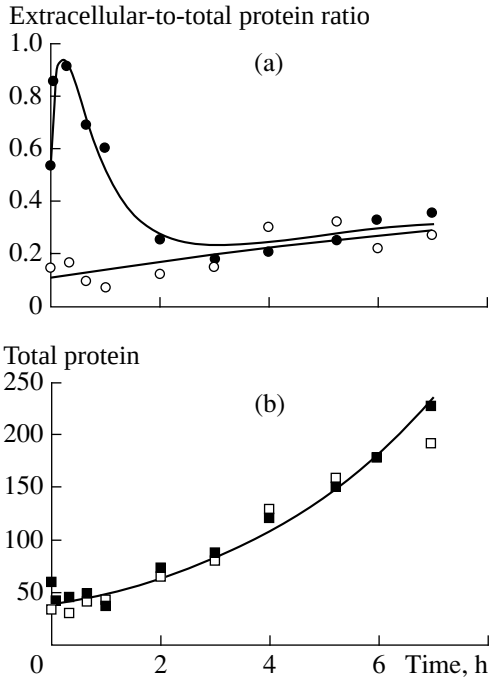

Fig. 2. Dynamics of (a) the extracellular-to-total protein ratio and (b) the total protein during the growth of *P. fluorescens* in a fresh medium (open circles and squares) and in the same medium with the added culture liquid filtrate (dark circles and squares).

NH_4Cl, 1; and glucose 2 (pH 7.0). The medium was supplemented with the following microelements (mg/l): $MgSO_4 \cdot 7H_2O$, 247; $CaCl_2$, 14.7; $MnSO_4 \cdot 4H_2O$, 0.4; $MnSO_4 \cdot 4H_2O$, 0.4; $ZnSO_4 \cdot 7H_2O$, 0.4; and $FeCl_3 \cdot 6H_2O$, 1 [10].

Protein dynamics was studied in the following experiment: Cells from a stationary-phase culture (24 h of growth; $OD_{600} = 1.5$; 7.5×10^8 cells/ml; 0.42 mg protein/ml) were washed with M9 medium on a 0.45-μm Millipore filter and suspended either in warm M9 medium or in the culture liquid (CL) of another culture to optical density $OD_{600} = 0.1$ (0.02–0.03 mg protein/ml). The cell suspension was dispensed in 1.5-ml aliquots into sterile Eppendorf tubes, which were then incubated at 30°C on a shaker (180 rpm). At regular intervals, three tubes were centrifuged at 9000 *g* for 2 min, and the supernatants and the cell pellets were analyzed for their protein content.

To obtain CL, the strain was grown in 250-ml flasks with 15 ml of M9 medium as described above. The inoculum was a 16-h culture added in an amount of 2–5 vol %. After 3–5 h of growth, when the exponential growth phase was reached, cells were removed from the culture by filtration through the Millipore filter.

Growth was monitored by measuring culture turbidity at λ = 600 nm on a Pye-Unicam SP-450 or Spekol spectrophotometer.

Protein concentration was determined by the Lowry *et al.* method [10] with bovine serum albumin as the standard.

The effect of proteinase K (BDH, United Kingdom) was studied by adding it to cells suspended in fresh M9 medium. The concentration of proteinase K was 0.2 μg/ml. R- and S-type cell variants for these experiments were grown to the exponential phase as described earlier [12]. The cell variants were used either separately or were mixed in various proportions.

RESULTS

The dynamics of batch growth was studied by growing pseudomonads either in a fresh medium (Fig. 1a, curve *1*) or in a medium with the added CL (Fig. 1b, curve *1*). In the fresh medium, the culture turbidity first decreased to reach a minimum at the 2nd hour of growth and then increased at a specific rate of about 0.25 h^{-1}. In the medium with the added CL, the culture turbidity exponentially increased from the very onset of incubation, indicating the absence of cell adhesion.

The dynamics of cellular and extracellular proteins during the growth of *P. fluorescens* in the fresh medium followed the exponential curves with specific rates of increase equal to 0.225 and 0.43 h^{-1}, respec-

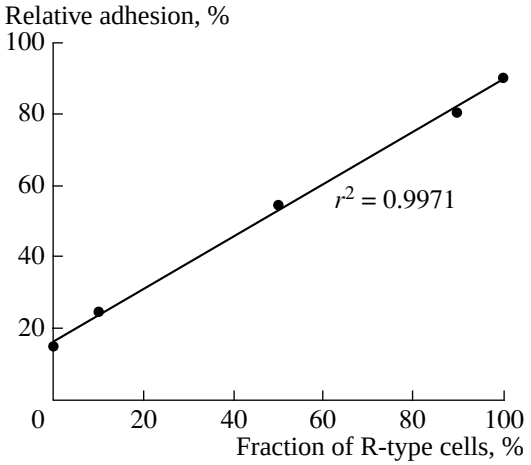

Fig. 3. The effect of the composition of the *P. fluorescens* population on cell adhesion.

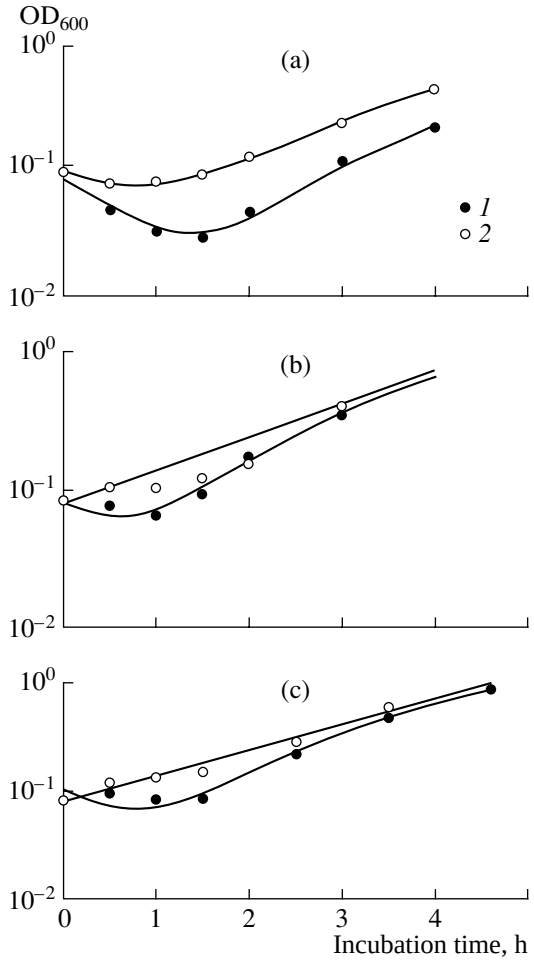

Fig. 4. The growth dynamics of (a) R-type cells, (b) S-type cells, and (c) a mixture of R- and S-type cells (R : S = 1 : 9) in M9 medium in the (*2*) presence and (*1*) absence of proteinase K.

tively (Fig. 1a, curves *2* and *3*). A decrease in the cellular protein content (which could be expected due to cell adhesion to the tube walls) was actually absent, since both free and adhered cells appeared in the pellet as a result of centrifugation. The specific rate of increase of the extracellular protein content was 1.7-fold higher than that of cellular protein. It is obvious, that the coincidence of the specific rates of increase of the culture turbidity and cellular protein (0.25 h^{-1}) was not accidental but rather due to the balanced metabolism of exponentially growing culture, as a result of which the protein content of cells was about the same (60–70% of the dry mass of cells). The increase in the content of extracellular proteins can be accounted for by the fact that daughter cells secrete more proteins into the medium than the mother cells. For this reason, the fraction of extracellular proteins in the total culture proteins tended to increase (Fig. 2a). In other words, cells introduced into a new medium excrete specific (?) proteins in amounts comparable with that of cellular proteins (10–30% of the cell mass). The physiological role of these extracellular proteins remains to be understood, although there is evidence that some extracellular enzymes hydrolyze (or transform) various compounds outside the cytoplasmic membrane.

In the medium with the added CL, the content of cellular protein drastically fell (by 20–80%) within 0.5 h after inoculation, but then rapidly increased to reach the control level over the next 2 h. The dynamics of extracellular protein was reciprocal: it first increased and then decreased to the control level. The sum of cellular and extracellular proteins followed the exponential curve with a specific rate of increase equal to 0.26 h^{-1} (Fig. 2b), which is close to the specific growth rate of pseudomonads calculated from the content of cellular proteins. Therefore, there are grounds to believe that the described dynamics of extracellular protein is due to its redistribution between cells and the medium rather than to its synthesis or digestion by proteases.

The extracellular-to-total protein ratio E was taken as a normalized estimate of the extracellular protein content for the following reasons: (1) the ratio E is limited from below and from above ($0 < E < 1$) by the material balance equations; (2) unlike the culture turbidity, the ratio E is independent of cell adhesion; and (3) the ratio E characterizes the contribution of the synthesis of extracellular protein to the total protein synthesis.

The addition of the CL obviously led to a release of the cellular protein into the medium, which explains the peak of extracellular proteins in Fig. 2a. The subsequent decrease in the content of extracellular proteins may be due to their reassociation with the cell wall and/or their proteolysis.

Differences in the adhesion of R- and S-type cell variants. In experiments with R and S cell variants,

adhesion was estimated as the ratio of the maximum decrease in the culture turbidity to the initial culture turbidity: $(OD_{ini} - OD_{min})/OD_{min}$. As is evident from Fig. 3, the relation between cell adhesion and the fraction of R cell variants in the inoculum was linear: when the fraction of R-type cells in the inoculum increased from 0 to 100%, the degree of cell adhesion increased from about 20 to about 90%. Therefore, the adhesive ability of cells and their colonial characteristic are likely related. Presumably, R-type cells have more powerful adhesins, which are responsible for cell adhesion to solid surfaces and other cells, than S-type cells.

The effect of proteinase K. To verify the suggestion that extracellular proteases are involved in cell desorption, we added commercial proteinase K to a bacterial culture and found that this protease considerably decreased cell adhesion (Fig. 4). The antiadhesive effect of proteinase K depended on the relative content of R and S variants in the cell population. The reversible adhesion of the population of R variants decreased under the action of proteinase K from 70 to 20%, while decreased from 20 to 0% in the case of the population of S variants. When the population contained 90–95% of S-type cells (this is a typical morphological composition of *P. fluorescens* populations [12]), cell adhesion and the degree of its inhibition by proteinase K were close to those of the population containing 100% of S-type cells.

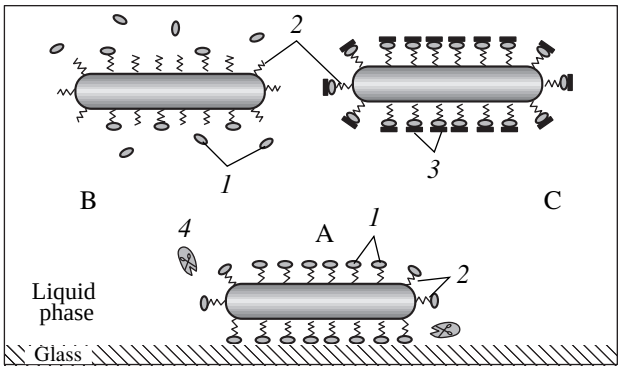

Fig. 5. Regulation of cell adhesion with the involvement of one adhesin (a glycoprotein complex) and two antiadhesins, AA_1 and AA_2. State A: a cell is attached to a surface with the aid of the adhesin molecules, representing unblocked glycoprotein complexes; antiadhesins are either inactive or are present in low concentrations. State B: the AA_2 molecules loss one of their two sticky ends under the action of a protease; the cell desorbs from the surface into the liquid phase. State C: the glycoprotein complex is reassociated against the background of an increased concentration of AA_1 in the medium; the adhesin molecules occurring on the cell surface are structurally intact, but inactive; as a result, the cell remains in the liquid phase. (*1*) Protein; (*2*) polysaccharide; (*3*) AA_1 (hydrocarbons); and (*4*) AA_2 (protease).

DISCUSSION

The hypothesis accounting for the putative mechanism of action of antiadhesins is schematically depicted in Fig. 5. Pseudomonads have adhesins of a glycoprotein nature on their cell wall [1, 2, 7]. In fresh media, cell adherence is regulated with the involvement of low-molecular-weight hydrophobic regulators and an extracellular protease, which cleaves one of the sticky ends of the adhesins [1–3]. When inoculated into a fresh medium, pseudomonads rapidly attach themselves to the wall of the cultivation flask through the action of the adhesins (state A). The culture liquid filtrate added to the medium contains antiadhesins, AA_1 (a mixture of hydrocarbons) and AA_2 (proteases). The hydrocarbons interfere with adhesion by blocking the sticky ends of the adhesins and favor the release of adsorbed cells by promoting their aggregation into large cell clusters. The proteases desorb the adsorbed cells by cleaving the adhesin molecules, due to which not only the cells but also a considerable portion of the adhesin glycoproteins are released into the liquid phase (state B). The rapid increase in the content of cellular proteins, along with the low level of extracellular polypeptides, suggests the existence of the equilibrium processes of the desorption and partial adsorption (reassociation with cells) of adhesins or their synthesis de novo. The glycoprotein complex is restored against the background of an elevated concentration of AA_1 in the medium. This results in the formation of intact but inactive adhesins on the cell surface (state C). They are resisitant to hydrolases, probably because AA is a protease inhibitor. In the control culture without added CL, cell adhesion is subject to delayed suppression by AA_1 and AA_2, whose accumulation in necessary amounts requires several hours.

Surprisingly, the contribution of adhesines to the total growth budget turned out to be fairly high judging from the fact cell release into the liquid phase was associated with solubilization of up to 80% of the cell proteins! Even if this number was somewhat overestimated (the Louri technique might give systematic error due to difference in the amino acid composition of adhesines and the calibration standard, and some part of the released proteins probably do not participate in cell adhesion), nevertheless it is clear that adhesines belong to major cell proteins. The high efficiency of extracellular proteases of *P. fluorescens* (rapid inactivation of adhesines at minimal accumulation of degradation products) is probably due to the fact that they act as endohydrolasese and break down only a few peptide bonds in each adhesine molecule.

The data presented are in agreement with the relevant data available in the literature. Pseudomonads have been reported to have protein-containing exopolysaccharides (EPSs) [5, 6], including the protein-containing adhesion factor [7]. The adhesin molecules are found both on the cell surface and in the culture liquid [2]. The phenomenon of reduction of cell adherence under the action of hydrolases has also been

described in the literature. Boyd and Chakrabarty showed the role of alginate lyase in the detachment of *Pseudomonas aeruginosa* cells [13]. London reported that the adsorbed cells of the bacteroid *Prevotella loescheii* detached from the colonized surface under the action of an extracellular protease when nutrient sources were exhausted [11]. Extracellular proteases were also found to be involved in the detachment of *Staphylococcus aureus* cells from the surface [14].

Various species of the genus *Pseudomonas*, *P. fluorescens* in particular, are characterized by the synthesis of exoproteases differing in their properties and stability [15–17]. The thermolability of some *P. fluorescens* exoproteases [16, 17] agrees well with the high thermolability of the antiadhesin found in the culture liquid of this pseudomonad [1, 2]. On the other hand, autoproteolysis [17] may be one of the mechanisms responsible for the rapid elimination of the respective antiadhesin. The autoelimination ability of AA_2 seems to be very important, since regulatory or sensory compounds must have a short life span, as is shown for hormones of higher organisms [18, 19]. The life span of signal molecules must not exceed the characteristic time of cell division, otherwise they will provide cells with incorrect information about the surrounding conditions.

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REFERENCES

1. Nikolaev, Yu.A. and Prosser, J.I., Extracellular Factors Affecting the Adhesion of *Pseudomonas fluorescens* Cells to Glass Surfaces, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 231–236.
2. Nikolaev, Yu.A. and Prosser, J.I., Some Properties of the *Pseudomonas fluorescens* Adhesin and Antiadhesin, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 237–242.
3. Nikolaev, Yu.A., Panikov, N.S., Lukin, S.M., and Osipov, G.A., Saturated C_{21}–C_{33} Hydrocarbons Are Involved in the Self-Regulation of *Pseudomonas fluorescens* Adhesion to a Glass Surface, *Mikrobiologiya*, 2001, vol. 70, no. 2, pp. 174–181.
4. Panikov, N.S. and Nikolaev, Yu.A., Growth and Adhesion of *Pseudomonas fluorescens* in a Batch Culture: A Kinetic Analysis of the Action of Extracellular Antiadhesins, *Mikrobiologiya*, 2002, vol. 71, no. 5, pp. 619–628.
5. Marshall, K.C., Mechanisms of Bacterial Adhesion at Solid–Water Interfaces, *Bacterial Adhesion: Mechanisms and Significance*, Savage, D.C. and Fletcher, M., Eds., New York: Plenum, 1985, pp. 133–162.
6. Christensen, B.E., Kjosbakken, J., and Smidsrod, O., Partial Chemical and Physical Characterization of Two Extracellular Polysaccharides Produced by Marine, Periphytic *Pseudomonas* sp. Strain NCIMB 2021, *Appl. Environ. Microbiol.*, 1985, vol. 50, no. 4, pp. 837–845.
7. Read, R.R. and Costerton, J.W., Purification and Characterization of Adhesive Exopolysaccharides from *Pseudomonas putida* and *Pseudomonas fluorescens*, *Can. J. Microbiol.*, 1987, vol. 33, pp. 1080–1090.
8. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor: Cold Spring Harbor Lab., 1989, vol. 3, p. A3.
9. Evdokimova, N.V., Dorofeev, A.G., and Panikov, N.S., Dynamics of Survival and Transition to a Dormant State in *Pseudomonas fluorescens* under Nitrogen Starvation Conditions, *Mikrobiologiya*, 1994, vol. 63, no. 2, pp. 195–203.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., Protein Measurement with the Folin Phenol Reagent, *J. Biol. Chem.*, 1951, vol. 193, pp. 265–275.
11. London, J., Bacterial Adhesins, *Annu. Rep. Med. Chem.*, 1991, vol. 26, pp. 239–247.
12. Nikolaev, Yu.A. and Mil'ko, E.S., Adhesive and Growth Properties of the R and S Variants of *Pseudomonas fluorescens*, *Mikrobiologiya*, 2000, vol. 69, no. 2, pp. 293–294.
13. Boyd, A. and Chakrabarty, A.M., Role of Alginate Lyase in Cell Detachment of *Pseudomonas aeruginosa*, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 2355–2359.
14. Fournier, B. and Hooper, D.C., A New Two-Component Regulatory System Involved in Adhesion, Autolysis, and Extracellular Proteolytic Activity of *Staphylococcus aureus*, *J. Bacteriol.*, 2000, vol. 182, no. 14, pp. 3955–3964.
15. Ruban, E.L., *Fiziologiya i biokhimiya predstavitelei roda Pseudomonas* (The Physiology and Biochemistry of Bacteria of the Genus *Pseudomonas*), Moscow: Nauka, 1986.
16. Hamamoto, T., Kaneda, M., Horikoshi, K., and Kudo, T., Characterization of a Protease from a Psychrotroph, *Pseudomonas fluorescens* 114, *Appl. Environ. Microbiol.*, 1994, vol. 60, no. 10, pp. 3878–3880.
17. Juan, S.M. and Cazzulo, J.J., The Extracellular Protease from *Pseudomonas fluorescens*, *Experientia*, 1976, vol. 32, no. 9, pp. 1120–1122.
18. Tepperman, J. and Tepperman, H.M., *Metabolic and Endocrine Physiology: An Introductory Text*, Chicago: Year Book Med., 1987.
19. Men'shikova, E.B., Zenkov, N.K., and Reutov, V.P., Nitric Oxide and NO Synthases in Mammals Occurring in Various Functional States, *Biokhimiya*, 2000, vol. 65, no. 4, pp. 485–503.