Study of the influence of external factors on the inheritance of forms of dissociants *Pseudomonas mandelii*

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Abstract. In the course of studying the phase variations of the *Pseudomonas mandelii* strain IB-Ki14, the pH of the medium was found to be the main factor inducing the switching of dissociants on LB medium. Dissociants of the *P. mandelii* strain IB-Ki14 are stably inherited at pH 5.0-6.0. Dissociant cells differed in antioxidant and protease-producing abilities. In general, matte colony cells had higher antioxidant and proteolytic activity. At the same time, the cells of the mucous colonies had a higher extracellular Arg-X protease activity compared to the matte ones and, accordingly, were able to hydrolyze a larger amount of protein from the nutrient medium. An analysis of the Arg-X protease activity of the dissociant cell fractions revealed differences at the level of 0.35 M NaCl fraction (loosely bound to the cell residue), which is quite possibly associated with the localization of proteolysis in a specific subcellular region, which allows spatial control of the content of regulatory proteins.

1. Introduction

Bacteria have developed various regulatory strategies to help them adapt to environmental changes. The phenotypic diversity of bacteria on the identical genetic basis (phase variations) is one of them. The phase variations are based on reversible changes in gene activity inherited in cell generations [1]. These processes are the result of a variety of genetic and epigenetic mechanisms: changes in chromatin structure, reversible rearrangements of the genome, modifications of nucleotides and proteins associated with DNA, maintenance of a certain level of transcription factors in a number of cell generations by means of the circulation of signals in cyclic gene systems. Proteolysis is directly involved in the implementation of some of these strategies, as regulation by proteolysis is universal and is involved in processes such as stress response, growth, division, cell cycle, cell development and differentiation, pathogenesis, biofilm formation, protein secretion [2-5]. Many bacteria produce extracellular proteinases, which can either be secreted into the extracellular environment or remain attached to the cell surface, making protein as an important source of nitrogen and carbon accessible to bacterial communities, [6-7]. The level of any protein in the cell is the result of a balance between synthesis and degradation [8]. It has been shown that regulation of proteolysis is beneficial in systems where a quick reaction is required, for example, under stress: the protein can be activated quickly without delays associated with the mechanisms of transcriptional and translational control, or the protein can be efficiently removed at a much faster rate than would allow simple dilution in a series of cell divisions. Proteolysis is a particularly effective regulatory mechanism in cases where reliability and irreversibility are required, for example, during passage through the cell cycle or in...
the process of cell differentiation [9]. The aim of the work was to study the phase variations of \textit{P. mandelii} with the identification of patterns of inheritance and phase switching. In addition, an analysis of antioxidant, proteolytic and endoglucanase activity was carried out.

2. Materials and Methods

The object of the study was Gram-negative bacteria \textit{Pseudomonas mandelii} placed in the group \textit{P. fluorescens}, strain IB-Ki14 (number in the All-Russian Collection of Microorganisms B-3250). In the experiments, the cells of the strain formed two types of colonies on plates: 1) matted, flat round opaque; 2) mucous, shiny, spreading over the surface. The cultures were cultivated on LB agar media [10] and an environment with a sprat hydrolyzate of the following composition: pancreatic sprat hydrolyzate 17.9 g/l; yeast extract 1.7 g/l; D (+) - glucose 3.0 g/l, microbiological agar 12.4 g/l, at a temperature of 28 °C. Colonies of two phenotypes of the strain are presented in figure 1. In the experiments, cells of 6-day-old colonies of two phenotypes were used.

![Figure 1. Bacteria \textit{Pseudomonas mandelii} placed in the group \textit{P. fluorescens}, strain IB-Ki14. 1. Matte colonies, flat, round, opaque; 2. Mucous colonies, shiny, spreading over the surface.](image)

To determine the endoglucanase activity, colony cells were transferred onto plates with LB medium containing 0.5% carboxymethyl cellulose (CMC). Cultivated 24 hours at 30 ° C. Poured with 0.1% Congo Red dye solution for 30 minutes. Washed 2 times with distilled water and poured with 1 M NaCl solution. After 15 min, the zones of CMCase activity were determined [11].

The total peroxide-destructive activity of bacteria was taken as an indicator of antioxidant activity. To determine the peroxide-degrading activity, colony cells were resuspended in Tris-HCl buffer (pH 7.0), cells were pelleted, resuspended and diluted in buffer to an optical density (D) of approximately 0.2 at a wavelength of 600 nm. 300 μl of 0.03% H$_2$O$_2$ was added to 300 μl of the obtained sample, and then after 3 minutes 300 μl of a 4% solution of ammonium paramolybdate. In the control, 300 μl of H$_2$O$_2$ and 300 μl of paramolybdate were mixed, and then 300 μl of the sample was added. The change in the content of hydrogen peroxide under the action of the antioxidant activity of the extract was determined as the difference between the content of peroxomolybdate in the control and the sample by absorption at wavelength of 405 nm on the MKMF-02 microphotocolorimeter, leading the reaction time to 1 min and $D_{600}$ to 1.

Fractionation of cell structures was performed as follows. Cells were washed with a medium of the following composition: 0.005 M MgCl2; 0.025 M KCl; 0.003 M CaCl2; 0.005 M NaCl; 0.01 M Tris-HCl, pH 6.8 and then the samples were aligned at D600 to approximately 1, which corresponds to 1.1 × 108 cells in 1 ml. Fractionation of cell structures was performed according to the method [12]. Cells were washed with 3% Triton X-100 in a medium of the following composition: 0.02 M triethanolamine (TEA), 0.005 M MgCl2; 0.025 M KCl; 0.003 M CaCl2; 0.005 M NaCl, pH 6.8; shaken for 30 min on a microshaker, followed by centrifugation at 4,000 rpm for 20 min to remove the cell membrane, after which the precipitate was washed twice in medium of the following
composition: 0.005 M MgCl2; 0.025 M KCl; 0.003 M CaCl2; 0.005 M NaCl; 0.01 M Tris-HCl, pH 6.8, followed by centrifugation under the above conditions. Concentrations below 3% of Triton X-100 did not remove the cell membrane of \( P. \) mandelii IB-Ki14. Next, sequential extraction of supramolecular cell structures was carried out. Cytoplasmic structures were extracted with 0.14 M NaCl, 0.01 M Tris-HCl buffer, pH 6.8. The fraction loosely bound to the cell residue (CR) was isolated by extraction of the pellet with 0.35 M NaCl, 0.01 M Tris-HCl buffer, pH 6.8. Then, the precipitate was fractionated by suspension in Tris-HCl buffer with 2 M NaCl, and the fraction tightly bound to the cell residue was obtained. The precipitate was a fraction containing a cell residue with a cell membrane. Subsequent extraction was performed with 6 M guanidine hydrochloride with 0.1% \( \beta \)-mercaptoethanol in Tris-HCl buffer. The amount of protein in the fractions was determined using the Bradford method with some modifications [13]. \( \text{Arg}-X \) proteolytic activity was evaluated by cleavage of \( \text{Arg}-X \) bonds in protamine rich in arginine protein. The activity of \( \text{Arg}-X \) proteolysis was expressed in nmol of arginine per second per 1 mg of protein [13]. Inhibitor-trypsin activity in fractions was determined by inhibition of trypsin [13]. The experiments were repeated three times, with at least three chemical repeats and two independent biological samples analyzed each time. Data are presented as arithmetic mean +/- standard deviation (SD). Research was conducted on the equipment of the center "Agidel".

3. Results and discussion

As mentioned above, in the experiments, cells of the \( \text{Pseudomonas mandelii} \) strain IB-Ki14 formed two types of colonies on dishes: 1) opaque, flat, round, opaque; 2) mucous, shiny, spreading over the surface. The growth pattern of the matte colonies changed on a medium with a sprat hydrolyzate at pH 6.0. The formation of concentric growth zones was observed, contributing to the rapid capture of the entire surface of the medium. This phenomenon is probably associated with the periodic formation of swarm, formed by cells with an excessive number of flagella.

The following factors were used to induce switching: change in cultivation temperature, pH of the medium, change in the content of amino acids and sugars in the medium, introduction of a source of methyl groups (S-Adenosylmethionine) into the medium. The experiments showed that the most significant factor in the inheritance and switching of colony phenotypes is the pH of the medium. Bacteria inherited both phenotypes on acidic media at pH less than 6.0. At pH 6.5, the switching frequency by plating increased (10% - cells of mucous colonies, 1% - matte), with increasing pH to 7.0 only matte colonies formed. In this case, the growth of colonies by plating of cells of the mucous colony began in 5-6 days.

![Figure 2](image.png)

**Figure 2.** Bacterial cultivation on plates with agar medium.
A. Matte colonies on LB medium. B. Mucous colonies on LB medium. C. The growth of opaque colonies on a medium with sprat hydrolyzate at pH 6.

It was found that dissociants differ in their ability to synthesize endoglucanases - enzymes that hydrolyze intramolecular glycosidic bonds of cellulose with the formation of short fibers. Activity
of endoglucanase, tested by the hydrolysis of carboxymethyl cellulose (CMC), was detected only in cells forming opaque colonies (figure 3).

![Image of CMC hydrolysis zones](image)

**Figure 3.** The formation of CMC hydrolysis zones by matte colony cells, detected using Congo Red.

The determination of extracellular Arg-X protease activity was carried out in order to evaluate the protease-producing ability of the colonies of dissociants of the *P. mandellii* strain IB-Ki14 strain (figure 4). At the same time, an analysis was performed on the protein content in nutrient media and on the surface of colonial cells (figure 5).

![Image of extracellular Arg-X proteolytic activity](image)

**Figure 4.** Extracellular Arg-X proteolytic (A) and trypsin inhibitor (B) activity. 1 – Matte colonies, 2 – Mucous colonies.

Both dissociants have been shown to have protease-producing abilities. At the same time, mucous colonies had a higher extracellular Arg-X protease activity compared to matte ones (figure 4A) and, as a result, the protein content of matte dissociants both in the nutrient medium and on the surface of the colonies was reduced (figure 5A, 5B). A parallel analysis of extracellular inhibitory activity against trypsin showed that the inhibitory trypsin activity was higher in matte colonies compared to mucous colonies (figure 4B), which may indicate the presence of a protease / inhibitor system, which plays a significant role in regulation of protein stability and interaction with the environment. As is known, protein function usually depends on its subcellular localization. Different approaches can be used to determine the localization of protein in the cell. We proposed a simple and effective method for isolating various compartments by fractionation by increasing the ionic strength of the solution [12]. Although biochemical analysis requires a violation of the anatomy of the cell, we have developed soft fractionation methods to separate the various components of the cell while maintaining their individual functions. Analysis of the Arg-X protease activity of the dissociant cell fractions (figure 6) revealed differences at the level of 0.35 M NaCl fractions (loosely bound to the cell residue), which may be associated with the localization of proteolysis in a specific subcellular region, which allows spatial control of the content of regulatory proteins (figure 7) and lead to the generation of cell asymmetry, therefore, determine the fate of the cell in the process of division [9].
Figure 5. Amount of protein in culture media (A) and on the surface of cell colonies (B). A: 1 – medium under a matte colony, 2 – medium under the mucous colony, 3 – medium-control. B: 1 – Matte colonies, 2 – Mucous colonies.

Figure 6. Arg-X proteolytic activity in cell fractions. 1 – Matte colonies, 2 – Mucous colonies.

Figure 7. The amount of proteins in cell fractions per cell volume taken in analysis (1.1 × 108 cells). 1 – Matte colonies, 2 – Mucous colonies.

Antioxidant activity both in the cells and in the fraction from the cell surface was higher in matte colonies (figure 8).
As a result of the experiments, it was shown that the matte colonies had a higher intracellular enzymatic activity compared to the mucous membranes (figures 4, 6, 8), on the basis of which it can be assumed that the antioxidant activity of dissociant cells is related to their metabolic rate.

4. Conclusions
One of the main factors for switching dissociants of the *P. mandelii* strain IB-Ki14 is the pH of the medium; according to this factor, switching patterns and inheritance of two forms are compiled. The dissociants showed pronounced differences in proteolytic and antioxidant activity, one of the dissociants has endoglucanase activity.

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