Fluorescence microscopy study of the effect of Esp1396I restriction-modification system proteins concentrations on protection against lambda phage.

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Abstract. Restriction-modification systems are among wide-spread mechanisms protecting bacteria from foreign DNA, such as DNA of bacteriophages and plasmids. The action of such systems is based on the presence of two enzymes — methyltransferase and restriction endonuclease, first one protects host DNA from degradation by modification and second one cleaves foreign unmodified DNA. In some cases bacteriophage DNA is modified faster than being degraded by restriction endonuclease. This process, called breakdown, leads to formation of modified bacteriophage progeny which is not sensitive to the action of restriction endonuclease and can eliminate whole “protected” bacterial population. There is a hypothesis which assumes that the overcoming of the protective action of restriction-modification system by a bacteriophage depends on the relative concentrations of the restriction-modification system proteins. To check this assumption we decided to perform fluorescence microscopy of individual living *E.coli* cells. For this purposes we use fluorescently-labelled type II Esp1396I restriction-modification system and also create an artificial system which allows to regulate the ratio of restriction-modification system proteins through the additional restriction endonuclease expression. Preliminary results showed an increase in protection with an increase in the restriction endonuclease concentration in cells.

1. Introduction

Due to increasing number of multidrug resistant bacteria the search of new antibacterial agents is extremely important. The use of bacteriophages for certain bacterial infections treatment seems to be a promising solution [1]. For this purpose it is very important to understand the mechanisms of bacterial protection systems against phages. This work provides deeper understanding of the mechanisms how phage overcomes the restriction-modification systems.

In nature, bacteria have to constantly protect themselves from bacteriophages and other foreign DNA. The restriction-modification (RM) systems are among the most wide-spread and efficient bacterial protection systems [2, 3]. The action of the type II RM systems is based on the activity of two proteins: a restriction endonuclease that cuts foreign DNA in specific recognition sites and methyltransferase which protects these sites by methylation. Thus, host DNA is always protected by methyltransferase while viral DNA is unmethylated and is degraded upon entry into the cell by restriction endonuclease. The concentrations of restriction-modification system enzymes are precisely regulated through various mechanisms [4, 5] to avoid host genome degradation by restriction endonuclease and bacteriophage genome modification by methyltransferase. But in case when viral
DNA is methylated before being degraded, all bacteriophage progeny becomes resistant to RM system (breakdown takes place).

There is a hypothesis [6] which assumes that this breakdown of the protection by bacteriophage can be not only stochastic, but also depend on the methyltransferase and restriction endonuclease concentrations ratio in each single cell. The main goal of current work is to find out whether the change in the relation of RM proteins in a single cell is able to change the protection level.

2. Materials and methods
To achieve the goal of this work we use fluorescently-labelled Esp1396I_fluo RM system described previously [7].

In addition to existing Esp1396I_fluo RM system we create an artificial system that allows to control and change the relative concentration of RM system proteins in bacteria. We create a plasmid, which makes it possible to express additional restriction endonuclease, and also to regulate the level of this expression. To achieve it, an arabinose promoter from pBadHisB plasmid was inserted into the pACYC184 plasmid by the Gibson Assembly method [8]. After that, the fluorescently-labeled restriction endonuclease from Esp1396I_fluo RM system fused with mCherry fluorescent protein was placed into the constructed plasmid under the arabinose-inducible PBAD promoter by the methods of restriction and ligation. To verify created system, new construction was sequenced [9].

E. coli strain DH5α cells were transformed with pBR322_Esp1396I_fluo and obtained pACYCBad_R_fluo plasmids according to standard transformation procedure.

For all experiments cells were grown in LB medium at 37 °C until OD600=0.4. Grown cells with constructed plasmid were placed under fluorescent microscope Nikon Eclipse Ti-E in a special microcultivation chamber described previously [7], with addition of arabinose for restriction endonuclease expression induction. Images were taken every 10 minutes into two channels (in transmitted light and in the fluorescence channel mCherry).

To check how the additional restriction endonuclease influences the RM system protection level we infect E. coli cells (without RM system; with Esp1396I_fluo RM system; with Esp1396I_fluo RM system and pACYCBad_R_fluo plasmid and different arabinose concentrations) with λ bacteriophage. For this experiment standard plaque assay was used.

3. Results and discussion
Analysis of fluorescence intensity of single bacteria showed that the amount of proteins of the RM system varies significantly from cell to cell, as well as their ratio (see Figure 1).

pACYCBad_R_fluo plasmid allowed us to change the ratio of RM system proteins. In cells harbouring pACYCBad_R_fluo plasmid we detect fluorescence in mCherry after induction of fluorescently-labeled restriction endonuclease (see Figure 2). In the presence of arabinose, cells die over time due to degradation of the cells genome by the restriction endonuclease.

We conducted an experiment to increase the expression of restriction endonuclease from constructed plasmid using different concentrations of the arabinose in addition to fluorescently-labeled RM system already existing inside the cells. Performed experiment showed an increase in protection even at the lowest concentration of the inducer by 1 order of magnitude (see Figure 3). With a further increase in the concentration of the inducer, the further improvement of protection against bacteriophage is practically absent, which can be described by saturation. In the future, it is planned to measure the concentrations of RM system proteins in individual cells, grown with different arabinose concentrations and use a fluorescently-labeled bacteriophage to observe the number of cell infections at different restriction endonuclease concentrations.
Figure 1. (a) Intensity distribution of restriction endonuclease (R) and methyltransferase (M) in individual *E.coli* cells; (b) Distribution of methyltransferase (M) to restriction endonuclease (R) ratio in individual *E.coli* cells; (c) Images of cells with pBR322_EspI396I_fluo plasmid in methyltransferase (left), restriction endonuclease (middle) and transmitted light channels (right).

Figure 2. (a) *E.coli* cells with pACYCBad_R_fluo plasmid in mCherry fluorescence channel (left picture) and transmitted light channel (right picture); (b) *E.coli* cells without any plasmids in mCherry channel (left picture) and transmitted light channel (right picture). Left fluorescent images have the same contrast.
Figure 3. Phage titer obtained on E.coli cells without RM system (black), with Esp1396I RM system (red) and with Esp1396I RM system and additional restriction endonuclease at different concentrations of arabinose (from blue to purple).

4. Conclusions

To check the hypothesis [6] which assumes that breakdown of the RM system protection by bacteriophage may depend on the methyltransferase and restriction endonuclease concentrations ratio in every single cell we shifted this ratio by increasing of restriction endonuclease concentration. As a result, we saw an increase in the level of protection by an order of magnitude compared with the RM system without additional restriction endonuclease induction. Such a result supports the correctness of this hypothesis.

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