The antiphage effect of ribonuclease binase in *Bacillus subtilis* population

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Abstract. Ribonuclease secreted by *Bacillus pumilus* (binase) exerts antiviral effect against viruses of animals and plants whereas its antiviral potential against viruses of bacteria has not been studied in deep. Here, the antiphage effect of binase was explored in a population of *Bacillus subtilis* since this species of *Bacillus* genus lacks genes homologous to binase. A bacteriophage active against *B. subtilis* was isolated from soil samples obtained from root zones of trees in urban park. It was shown that exogenously applied binase interfered with phage reproduction in *B. subtilis*. Binase synthetized endogenously from a chromosomally integrated gene in a recombinant strain of *B. subtilis* reduced page titer by two orders of magnitude in comparison to the parent *B. subtilis* strain without binase gene. The antiphage effect of binase was more pronounced when the number of phage particles was small. Therefore, it was found that expression of ribonuclease binase in *B. subtilis* can limit phage infection.

1 Introduction

Bacteriophages (phages) are the most abundant biological agents on Earth which infect bacterial cells only. They regulate the number of bacteria in biosphere and drive bacterial evolution being important participants in microbial communities [1]. As a natural bio-control agents they disrupt up to 40 % bacterial cells every day [2]. Despite the wide diversity of bacteriophages, they all consist of nucleic acid enclosed in a protein shell called a capsid, which protect the genetic material and help them to deliver their DNA or RNA genome into the host cell. Phages have been discovered in all niches where bacteria live, but their global impact on existing microorganisms, and most importantly their biotechnological potential, remains underestimated. Current bacteriophage research is mainly aimed at developing phage therapy to treat bacterial infections [3]. However, despite the significant application potential of phage, accidental and uncontrolled viral contamination can pose serious risks. Bacteriophages can increase the risk of bacteria developing antibiotic resistance, enhancing bacterial virulence and pathogenicity through lysogenic conversion, potentially leading to detrimental outcomes for human health [4]. In anthropogenic biotechnological processes, notably in the food and pharmaceutical industries, bacteriophages disrupt fermentation processes by killing bacteria thereby affecting the quality of end products [2, 5].

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In response to frequent bacteriophage attacks, bacteria continuously develop and refine strategies to protect themselves against phage infection [6]. The first line of defense for a bacterial cell is generally considered to be preventing phage penetration into the host cell. The emergence of various protective layers, modification of surface receptors, and the development of competitive inhibition are just a few of the defense mechanisms that block the possibility of viral attachment to the cell [7]. In the case of cell infection, numerous mechanisms exist that target the destruction of phage genetic material. Specific nuclease enzymes recognize and cleave foreign DNA or RNA, depriving bacteriophages of the ability to replicate. This defense strategy includes both classical restriction-modification systems as well as newer systems such as DISARM and CRISPR-Cas [6, 8]. Cell death or abortive infection represents a last resort, wherein an infected cell sacrifices itself to preserve the entire bacterial population [9].

Enzymes that degrade nucleic acids actively participate in cell defense against viral invaders. For example, ribonucleases can directly degrade phage RNA as part of type III and type IV CRISPR-Cas systems [8]. Furthermore, within abortive infection ribonucleases act as toxins that eliminate phage-infected cells [9]. Previously, it was shown that binase, a guanyl-preferring low-molecular-weight ribonuclease secreted by *Bacillus pumilus*, exerts a profound antiviral effect against both RNA and DNA viruses of animals, namely influenza virus, reovirus, rhinovirus, rabies virus [10]. However, the antiviral role of binase in bacterial population was not yet studied in deep. The aim of this work was to elucidate the effect of binase on phage infection of *Bacillus* cells.

2 Materials and methods

2.1 Bacterial strains and cultivation

B. subtilis 168 (*trpC2*) was used as a host strain for isolation, propagation and enumeration of bacteriophage. Its derivative, *B. subtilis* 168-bin (*trpC2 bmr3::bin+brst*), was used to study the effect of ribonuclease binase on phage infection. It comprises a genetic cassette consisting of full-length genes for binase and its intracellular inhibitor barstar which was introduced into *bmr3* locus of *B. subtilis* 168 chromosome. Barstar protects bacterial cells from toxic action of binase.

The strains were cultured in GRM broth (g/L: pancreatic hydrolysate of fish meal – 8, peptone – 8, NaCl – 4; State Research Center for Applied Biotechnology and Microbiology, Obolensk, Russia) supplemented with 0.6% or 1.7% agar-agar when needed and incubated at 37 °C. The growth of bacteria was measured spectrophotometrically at the wavelength 590 nm and expressed as optical density units (OD_{590}).

2.2 Binase preparation and activity determination

Binase (12.3 kDa, pI 9.5) preparation with enzymatic activity of 1.2×10^6 units per milligram of protein was used. Ribonuclease activity was determined using a modified Anfinsen method based on the amount of acid-soluble products which are formed during 0.1 % RNA hydrolysis by 1 mL of enzyme-containing solution for 1 h at 37 °C.

2.3 Bacteriophage enrichment from soil

Soil samples were collected from public parks in Kazan, Russia. A laboratory strain of *B. subtilis* 168 was used as a host for enrichment of bacteriophages from the soil samples.

Initially, soil samples (1 g) were suspended in sodium phosphate buffer (g/L: NaCl – 8, KCl – 0.2, Na_2HPO_4 – 1.44, NaH_2PO_4 – 0.24, pH 7.4) and incubated for 90 min with periodical mixing by inversion. Then, they were mixed with 10 mL of nutrient broth and 0.2 mL of an overnight culture of *B. subtilis* 168. incubated with shaking (160 rpm) at 37 °C for 24 h, centrifuged at 3500× *g* for 10 min. Subsequently, the supernatants were filtered through a polyethersulfone 0.22 μm filter (Millipore, USA) and stored at 4 °C.

2.4 Bacteriophage detection by drop method

A drop method was used for a rapid detection of *B. subtilis*-specific bacteriophages in the samples. 200 μL of the overnight culture of *B. subtilis* 168 were spread on the surface of nutrient agar and left for absorption for 15 min. Afterwards, 10 μL of phage suspension was placed on upper part of the Petri plate. The plate was slowly tilted to let the drop flow down and incubated at 37 °C for 24 h. The presence of bacteriophages in the samples was evaluated by the formation of clear lysis zones on the way of the dripping drop.

2.5 Bacteriophage titer determination

Phage titers were estimated using a double-agar overlay plaque assay on host strain of *B. subtilis* 168. The phage lysate was serially 10-fold diluted in nutrient broth. 100 μL of an appropriate dilution of bacteriophage lysate was mixed with 200 μL of bacterial overnight culture, and 3 mL of molten soft agar, then the mixture was poured onto prepared nutrient 1.7 % agar plate. The plates were incubated at 37 °C for 16-18 h and plaques were counted. Phage titer was expressed as a number of plaque-forming units per mL (pfu/mL) of phage-containing sample.

2.6 Bacteriophage propagation

Overnight culture of *B. subtilis* 168 was diluted with a nutrient broth and incubated for 4 h at 37 °C until the mid-log phase ($OD_{590} \approx 0.6$). Then, bacterial suspension was mixed with bacteriophage-containing filtrate at the ratio 1:10 and incubated at 37 °C for 24 h. The bacteria–phage mixture was centrifuged at 3500× g for 15 min. The supernatant was then filtrated through a polyethersulfone 0.22 μm filter (Millipore, USA) and analyzed on double-layer agar plates of the *B. subtilis* 168. The procedure was repeated for several times until the uniform phage suspension with a titer of 10^6-10^8 pfu/mL was achieved.

2.7 Bacteriophage infection assay

Overnight grown bacteria were diluted by fresh nutrient media to ~10^8 colony-forming units (cfu) per milliliter and infected by phage at multiplicity of infection (MOI) equal to 0.1 or 0.01. The value of MOI was calculated as the ratio of the number of phage particles to bacterial cells (pfu/cfu). In experiments with pure binase, the enzyme was added to the bacteria-phage mixtures at a final concentration of 100 μg/mL. The mixtures were incubated for 1-24 h. After the growth of bacteria was measured, cells were pelleted by centrifugation at 3500× g for 10 min, and supernatants were used for the determination of phage titers and binase activity. Uninfected strains were used as a negative control.

2.8 Spot assay

Two strains of *B. subtilis*, 168 and 168-bin, were grown overnight. Then, 0.2 mL of each culture was mixed with 3 mL of molten soft agar and poured onto prepared nutrient agar plate. The plates were incubated for 30 min at room temperature. The phage lysate was serially 10-fold diluted in nutrient broth. 2 μL of each dilution was pipetted onto a lawn of *B. subtilis* cells in the top agar. After 16-18 h of incubation at 37 °C the lytic activity of the bacteriophage against the two *B. subtilis* strains was evaluated by observing the appearance of formed spots.

2.9 Statistical analysis

Statistical processing and visualization of quantitative data were performed using GraphPad Prism 8 software (GraphPad Software, USA). Data on histograms were presented as mean (M) ± standard deviation (SD). The Kruskal-Wallis test followed by Dunn post-hoc test was used to compare the parameters in groups. Differences were considered statistically significant at $p < 0.05$.

3 Results

3.1 Isolation of bacteriophage active against *B. subtilis*

To isolate bacteriophages capable of infecting *B. subtilis* from soil a conventional phage-enrichment method was used. Five soil samples from different parts of an urban park were analyzed. Samples №1 and №2 were obtained from root zones of trees. Samples №3 and №4 were characterized by high content of sand and decaying plant matter, respectively. Sample №5 was taken near to a pond. After the samples were incubated with a nutrient broth supplemented with an overnight culture of *B. subtilis* 168, the presence of host-specific bacteriophages in them was assessed by a drop method. Bacteriophages infecting *B. subtilis* were found in all soil samples except for the sample №5 (Fig. 1). The sample №2 contained the highest concentration of virus particles, and it was used for bacteriophage isolation and propagation. The isolated bacteriophage formed clear plaques with translucent halo zones in double agar overlay assay with *B. subtilis* as the indicator host.

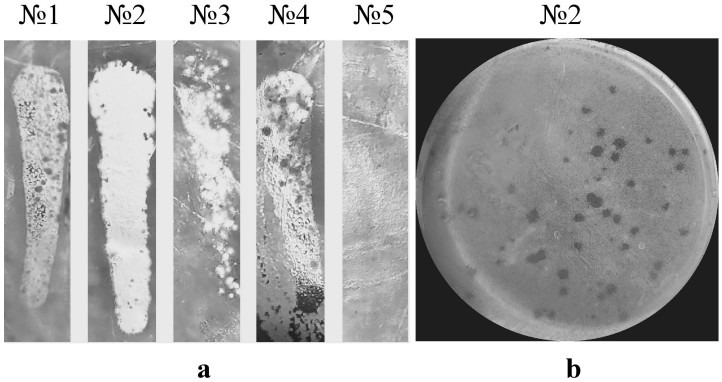

Fig. 1. Isolation of bacteriophages infecting *B. subtilis* from soil. (a) Presence of B. *subtilis*-specific bacteriophages in the soil samples №1-5 tested by the drop method. (b) Plaques formed by bacteriophages from the soil sample №2 on the lawn of *B. subtilis* 168.

3.2 Examination of the antiphage activity of exogenous binase

To check the ability of binase to influence phage infection in *B. subtilis*, bacteria were incubated with phage at MOI 0.01 in the presence or absence of binase for 6 h and their proliferation was estimated. This relation gives a 100-fold excess of the number of bacterial cells over viral particles in the medium and allows multiple cycles of infection. The multiplication of bacteriophage in *B. subtilis* culture brought about the decrease in the growth of bacteria by 46 % (Fig. 2). Upon addition of binase, the growth of phage-infected *B. subtilis* cells was lowered by 21 % only (Fig. 2). Phage was less effective in the presence of binase than its absence, therefore, binase can exert anti-phage activity by interfering with phage reproduction in *B. subtilis* cells.

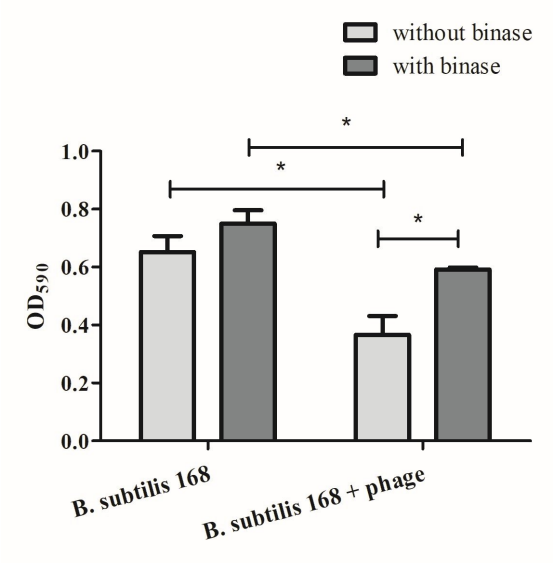

Fig. 2. Changes in the optical density (OD_{590}) of *B. subtilis* culture grown in the nutrient medium for 6 h in the presence of phages (MOI 0.01) and/or binase (100 μg/mL).

3.3 Examination of the antiphage activity of endogenous binase

To analyze the anti-phage activity of endogenously synthesized binase, a *B. subtilis* strain 168-bin bearing a binase gene on its chromosome was used. The efficacy of the phage infection of *B. subtilis* cells expressing binase gene in comparison to the parent strain was investigated through plaque formation in a spot assay. The bacteriophage was able to lyse both parent strain and its binase-producing derivative but at different titers (Fig. 3). In the former case, completely clear spots were detected at phage titers more than 10^2 pfu/mL and single plaques were seen at titer 10^1 pfu/mL. In the latter case, clarified spots were observed at phage titers 10^4-10^5 pfu/mL, single plaques – at titer 10^3 pfu/mL, and no plaques at titers 10^1-10^2 pfu/mL. Therefore, upon binase production bacteria resist infection by phages but only when the number of phage particles is small.

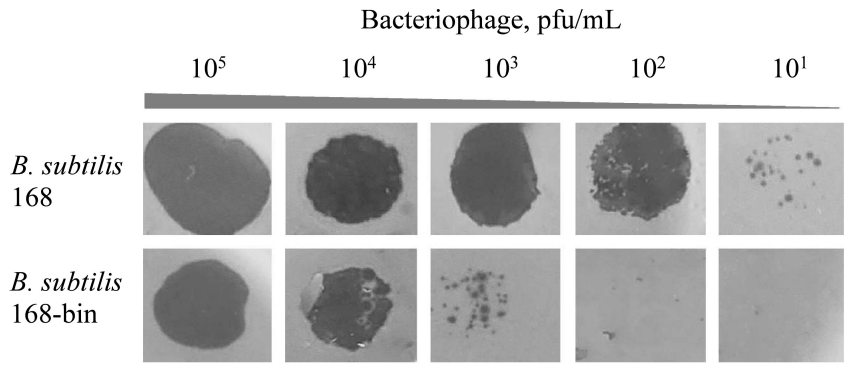

Fig. 3. Spot assay for evaluating the efficacy of bacteriophage to infect *B. subtilis* strain 168-bin expressing binase gene as compared to the parent strain *B. subtilis* 168.

To investigate the effect of binase biosynthesis on phage propagation in *B. subtilis* cells further, bacteria were infected by phage at MOI 0.1. This relation gives a 10-fold excess of the number of bacterial cells over viral particles in the medium, leading to around 9.5 % of infected cells in bacterial population. It ensures that bacteria can multiply and produce ribonuclease while phages can make multiple cycles of infection. After 24 h incubation, phages multiplied to the titer of ~10^9 pfu/mL in *B. subtilis* strain 168 causing the 1.6-fold decrease in the optical density of the culture and elimination of its extracellular ribonuclease activity (Table 1). In binase-producing strain *B. subtilis* 168-bin the phage titer was 1.7 order less than in parent strain, bacterial growth and ribonuclease activity were affected only slightly (Table 1). Therefore, expression of binase in *B. subtilis* can limit phage infection.

Table 1. Bacteriophage reproduction in *B. subtilis* strain expressing binase gene.

Strain	Growth, OD_{590}	Binase activity, units/mL×h	Phage titer, pfu/mL
without phage			
B. subtilis 168	2.65±0.05	150±13	n/a
B. subtilis 168-bin	2.64±0.06	910±68	n/a
with phage			
B. subtilis 168	1.63±0.05	4±1	1.12±0.07 × 10^9
B. subtilis 168-bin	2.32±0.08	628±47	6.70±0.35 × 10^6

4 Discussion

Bacteria of genus *Bacillus* find their application in agriculture as plant growth promoters and biocontrol agents, and in biotechnologies as producers and hosts for production of biologically active compounds [11, 12]. Bacteriophages capable of infecting *Bacillus* species

can interfere with these processes making them inefficient. In this work, the role of the ribonuclease binase in the resistance of *Bacillus* cells to phage infection was studied. The antiphage effect of binase was assessed by comparing the responses of the two *B. subtilis* strains to phage infection, namely the wild type strain of *B. subtilis* which has no genes homologous to binase gene and the wild-type-derived recombinant strain with the chromosomally introduced binase gene.

Since *B. subtilis* is a soil-living saprotroph, different soil samples were collected for the isolation of its bacteriophages. The presence of a large number of *Bacillus*-infecting bacteriophages in soil samples collected from the tree root zone was not surprising because *B. subtilis* is commonly found in association with plants and their rhizosphere [13]. The bacteriophage isolated from these samples formed clear plaques on the lawn of *B. subtilis* indicating the lytic type of interaction (Fig. 1). Translucent halos surrounding the plaques indicated the presence of polysaccharide depolymerases degrading exopolysaccharides of host cells during infection like in the case of φAB6 phage [14].

Assessment of binase ability to affect bacteriophage reproduction in *B. subtilis* cells was studied using both exogenously applied and endogenously produced binase. It was shown that infection of bacteria by phage in the presence of binase was about two times less effective than without the addition of the enzyme (Fig. 2). The biosynthesis of binase protected *B. subtilis* cells from phage infection especially when the number of virions was small (Fig. 3, Table 1). The phage multiplied in binase-producing cells to the titers almost two orders of magnitude less than achieved in the wild type cells (Table 1). Meanwhile the level of extracellular ribonuclease activity was changed only slightly mostly at the cost of a non-specific high-molecular weight ribonuclease Bsn secreted by *B. subtilis* itself (Table 1). Therefore, it can be concluded that biosynthesis of binase in the recombinant strain of *B. subtilis* is not affected by the phage infection but the lytic activity of the bacteriophage is reduced in the presence of binase.

Thus, binase is able to make phage less effective against *Bacillus* cells. Being the small cationic protein binase can interact with the negatively-charged molecules on the cell surface of bacterial cells interfering with phage absorption. These molecules can be represented by teichoic acids in the cell wall of gram-positive bacteria which participate in interaction with bacteriophages [15]. Also, binase can penetrate bacterial cells and degrade RNA molecules impairing phage reproduction inside bacterial cells or even leading to cell death like in the case of some toxin-antitoxin systems which activation leads to the abortive phage infection [9] Though the exact mechanism underlying antiphage effect of binase still remains to be uncovered, the obtained data indicate that binase can be used in the development of antiphage approaches.

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