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EXPERIMENTAL ARTICLES

Influence of the Chitosan Oligomer on the Phage Particles and Reproduction of Phage 1-97A in the Culture of *Bacillus thuringiensis*

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Abstract—The causes of bacteriophage 1-97A inactivation by the chitosan oligomer with a polymerization degree of 15 and the influence of the oligomer on the phage reproduction in the culture of *Bacillus thuringiensis* subsp. *galleriae*, strain 1-97, were studied. The study of the inactivation kinetics showed that, in 1 h, virtually all chitosan was bound to the phage particles, causing, as evidenced by electron microscopy, DNA release from the phage head, destruction of the phage particles, and agglutination of the phage particles or of their tails in the region of the basal plate. High-polymeric chitosan caused more pronounced destruction of the phage particles than the oligomer. It was established that chitosan prevented the production of complete phage particles. One of the mechanisms of such an influence may be the production in the presence of chitosan of phage particles devoid of DNA.

Key words: chitosan, bacteriophage, *Bacillus thuringiensis.*

Chitosan is known to prevent the development of phage infections in cultures of various microorganisms. In particular, it was shown that chitosan prevented the infection caused by phage 1-97A in the culture of *Bacillus thuringiensis* [1]. The study of the influence of different chitosan derivatives showed that one of the causes of the suppression of infection is the inactivation of mature phage particles by chitosan. However, the mechanism of phage inactivation remains to be understood. On the other hand, inactivation is not the only cause of the suppression of phage infection. Thus, it was established that chitosan preparations with a high degree of polymerization (PD) have a high inactivating activity, but insignificantly inhibit the infectious process. At the same time, chitosan oligomers, which cause insignificant inactivation of the phage particles, efficiently inhibit the reproduction of bacteriophage 1-97A in the bacillar culture. It was suggested that chitosan might influence bacteriophage reproduction, but no direct evidence supporting this supposition was obtained [2].

The task of this work was to study the causes of inactivation of extracellular phage particles by chitosan and the influence of chitosan on the reproduction of bacteriophage 1-97A. For the latter purpose, a chitosan oligomer (PD = 15) was used since the degree to which the infection is suppressed by this derivative significantly exceeds the level of inactivation of phage particles that it causes [2]. This circumstance offered the possibility of a differentiated assessment of the two effects of chitosan to determine their roles in the inhibition of the phage infection of the bacillar culture.

MATERIALS AND METHODS

The properties of *Bacillus thuringiensis* subsp. *galleriae*, strain 1-97, the virulent phage 1-97 A, the chitosan oligomer with a PD of 15, and the experimental conditions were described earlier [1, 2]. A single phage reproduction cycle was studied according to the method described by Adams [3]. The adsorption rate constant was calculated by the formula cited in [3]:

$$k = 2.3N^{-1}t^{-1}\log P_0/\text{P, ml/min},$$

where N is the number of bacterial cells; t is time, min; P_0 is the initial number of the phage particles at the beginning of the period t; and P is the number of unadsorbed phage particles at the time t.

To study the effect of chitosan on phage reproduction, it was introduced into a sample of phage-infected cells 1 h after the infection. The final chitosan concentration was 100 μg/ml. No chitosan was added to the control sample. The phage titer in the test and control samples was determined by the agar layers method [2] 1 and 3 h after the addition of chitosan.

In the other experimental series, the chitosan oligomer at the same final concentration (100 μg/ml) was added to the phage particle suspension, and the phage titer was determined 1 and 3 h after the addition of chitosan. In some experiments, high-polymeric chitosan

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Effect of chitosan on the development of infection and the infectivity of mature phage particles*

Incubation time after chitosan addition, h	Phage titer in the infected culture supernatant		Phage titer in the phage particle suspension	
	control without chitosan	with chitosan, 100 μg/ml	control without chitosan	with chitosan, 100 μg/ml
0**	4.0×10^7	4.0×10^7	6.7×10^7	6.7×10^7
1	2.0×10^8	7.2×10^5	6.7×10^7	3.5×10^5
3	4.0×10^{10}	4.6×10^5	6.7×10^7	3.5×10^5

* The results of typical experiments are presented.

** Immediately before the addition of chitosan.

(PD = 250) was added to the phage particle suspension, and the suspension was incubated as described earlier [2].

To study the effect of chitosan on the structure of the phage particles, electron microscopy was used. These studies were conducted using a JEM-100CX electron microscope at a 50000× magnification. The preparations were negatively stained with a 2% solution of phosphotungstic acid, pH 7.0. The structure of the phage particles produced in the phage-infected bacillar culture in the presence of chitosan was studied in the same way. Staining of the phage particles with homologous antiphage serum and their further study by electron microscopy were carried out as described earlier [4].

To assess the effect of chitosan on the bacillar cells, the culture was introduced into a liquid M9 medium to a final cell concentration of 5×10^8 and incubated on a shaker at 260 rpm for 2.5 h at 32°C. The chitosan oligomer was then added to the test samples, and incubation was continued under the same conditions. The number of viable cells in the experiment and in the control was determined 1 and 3 h after the addition of chitosan. The colonies were counted after plating tenfold serial dilutions onto petri dishes with an agarized LB medium.

RESULTS

A preliminary study of the kinetics of the infectious process showed that the phage adsorption on the bacillar cells was completed within 45 to 60 min. About 84 to 97% of the phage particles were adsorbed within this time. The adsorption rate constant was 1.5×10^{-9} to 6.2×10^{-10} ml/min, which attested to the optimum conditions for the infection process. The study of a single cycle of the bacteriophage 1-97A reproduction in the culture of *B. thuringiensis* showed that the latent period of infection constituted 45 min; the cell lysis period lasted 2.5 h. The phage yield was 90 to 100 particles per cell.

Proceeding from these data, the chitosan oligomer was introduced 1 h after infection, i.e., after the phage adsorption had been accomplished. The first determination of the phage titer in the infected culture was carried out 1 h after chitosan introduction, i.e., upon the completion of the latent period; the second determination was performed 3 h after the addition of chitosan, i.e., in the period of maximum phage accumulation in the control. The influence of chitosan on the phage particles and bacillar cells was studied in the same time intervals.

It was established that, during the experiment, the number of bacillar cells in the control sample did not increase; neither did it decrease in the test sample (the data are not shown). This indicates that the chitosan oligomer does not decrease the viability of *B. thuringiensis* cells, and, consequently, the suppression of the phage reproduction is not related to the negative effect of chitosan on the bacillar cells.

The results of the study of the influence of chitosan on the phage particles and phage reproduction are shown in the table.

It is obvious that chitosan inactivated phage particles. It is important to note that the inactivation was completed in the first hour of phage incubation with chitosan. Later, the titer of the phage suspension did not change.

Phage titration in the supernatant of the infected bacillar culture showed that the phage actively reproduced in the control and did not reproduce in the test samples supplemented with chitosan. A significant (approximately 50-fold) drop in the phage titer in the test sample in the first hour of incubation of the infected culture with chitosan is noteworthy. The absence of the increase in the phage titer in the test sample after 3 h of incubation shows that chitosan suppresses phage 1-97A reproduction.

Electron micrographs of phage 1-97A particles incubated with the chitosan oligomer are presented in Fig. 1. Along with an insignificant number of outwardly intact phage particles (Fig. 1a), we observed particles whose electron-dense material underwent structural reorganization, condensing at the center of the head (Fig. 1b) or disposing eccentrically, nearer to the tail (Fig. 1c). The heads of most particles appeared to be empty (Fig. 1d). These photographs may represent different stages of DNA release from the phage head caused by chitosan. Separate tails (Fig. 1e) and empty heads occurred frequently (Fig. 1f), which indicates the destruction of the phage particles by chitosan. In addition, the agglutination of particles or tails in the region of the basal plate was observed (Fig. 1g).

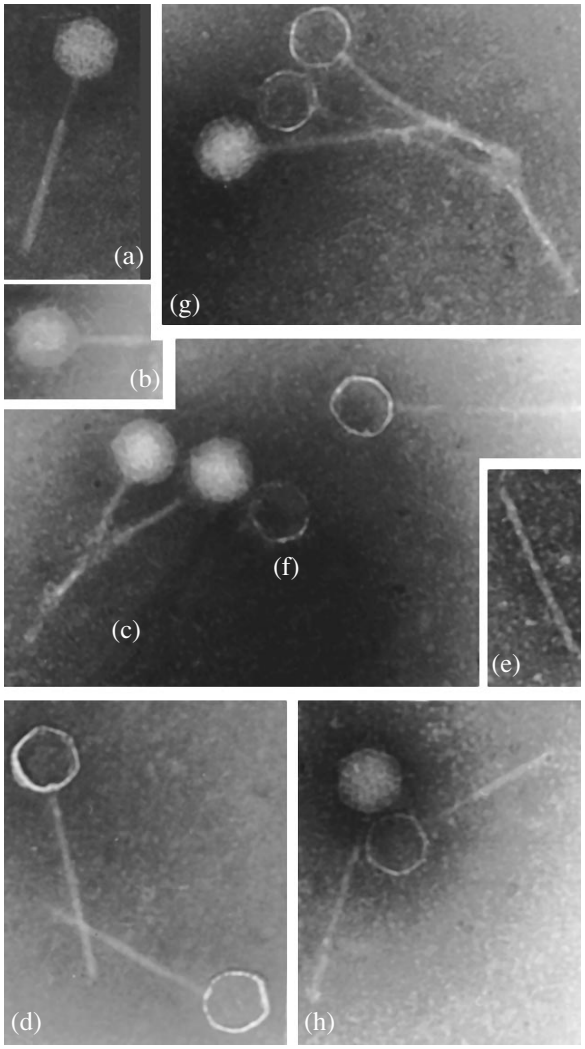

Fig. 1. Inactivation of bacteriophage 1-97A by the chitosan oligomer with a DP of 15. See explanations in the text. Magnification, 100000×.

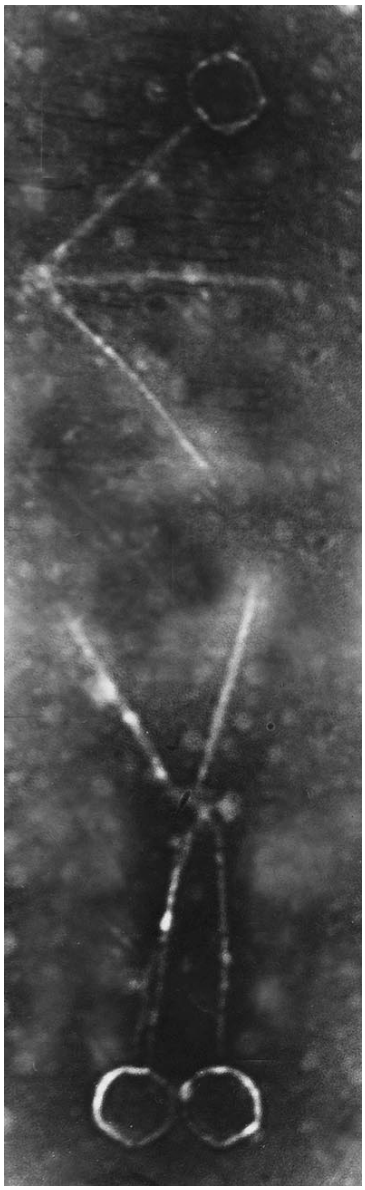

Fig. 2. Inactivation of bacteriophage 1-97A by high-polymeric chitosan with a DP of 250. Magnification, 120000×.

Upon the exposure of the phage particles to high-polymeric chitosan, we observed phage particles with empty, DNA-free heads, separate empty heads, and separate tails of phage particles, as well as the agglutination of particles or tails in the basal plate region (Fig. 2).

Electron-microscopic studies of particles stained with a homologous antiserum after phage incubation with the chitosan oligomer revealed no changes in the antigenic structure. The antibodies uniformly covered the whole surface of the phage particles (data not presented), as in the case of staining intact phages [4].

Electron-microscopic studies of the structure of the phage particles produced in the infected bacillar culture in the presence of the chitosan oligomer revealed a picture that was absolutely different from that observed upon the inactivation of the phages. In this case, we observed an approximately equal number of outwardly intact phage particles (Fig. 3a) and of DNA-free particles whose structure looked, however, unchanged and which had well-defined capsomers (Fig. 3b) [5].

DISCUSSION

It was suggested earlier that chitosan is capable of preventing the development of phage infection in several ways, namely by decreasing the number of viable cells in the microbial culture where the phage reproduction occurs, by inactivating daughter phage particles, and by suppressing bacteriophage reproduction [6]. The use of different chitosan derivatives showed that the relative significance of each of these factors

depends on the chemical structure of chitosan, particularly on its polymerization degree [2]. Therefore, in this work we used a chitosan oligomer with a PD of 15, and studied its influence on three parameters of the infectious process: on the accumulation dynamics of the infective phage in the infected culture, on the degree of inactivation of mature phage particles, and on the number of viable cells in the bacillar culture.

It turned out that the chitosan oligomer did not decrease the viability of the bacillar culture. Thus, the number of bacterial cells sustaining the bacteriophage reproduction was not the factor limiting the development of phage infection.

On the other hand, the results of this work once again confirmed that inactivation of the phage particles by chitosan is an important factor suppressing the infection. The study of the inactivation kinetics first undertaken in our work allowed substantial details of the process to be revealed. Inactivation proceeded quickly enough and was completed within the first hour of incubation of the phage suspension with chitosan. The fact that no further decrease in the phage titer occurred during the subsequent incubation of the phage suspension with chitosan is likely to mean that virtually all chitosan was irreversibly bound to the phage particles, causing, in a short period of time, the changes shown in Figs. 1b–1g and determining a two-orders-of-magnitude decrease in the phage titer.

An important result of the interaction of chitosan with phage particles was the aggregation of the particles or their tails in the basal plate region. It can be suggested that chitosan is an agglutinating agent. It cannot be ruled out that it is the binding of chitosan to the basal plate, where receptor-recognizing phage proteins are localized, that triggers the DNA release from a phage particle in the absence of a host cell. Whether this is determined by the fact that glucosamine residues are a crucial structure of the phage receptor or a nonspecific interaction occurs between chitosan and the basal plate remains to be clarified.

In our previous work, it was shown that high-polymeric chitosan preparations inactivate phage 1-97A particles to a greater degree than their oligomer fragments [2]. The electron-microscopic studies undertaken in the present work revealed a possible cause of this difference. The comparison of Figs. 1 and 2 shows that the high-polymeric chitosan caused much more pronounced destruction and aggregation of the phage particles than the chitosan oligomer.

Thus, the cause of inactivation of phage 1-97A particles by chitosan is the initiation of a DNA release by phage particles in the absence of host cells, destruction of phage particles, and the formation of their aggregates. It should be noted that the process of inactivation of phage T2 by the chitosan oligomer with a PD of 19 occurred in a different way; in particular, it was not accompanied by DNA release from the phage head [7]. The inactivation mechanism seems to depend on the structural features of a particular phage.

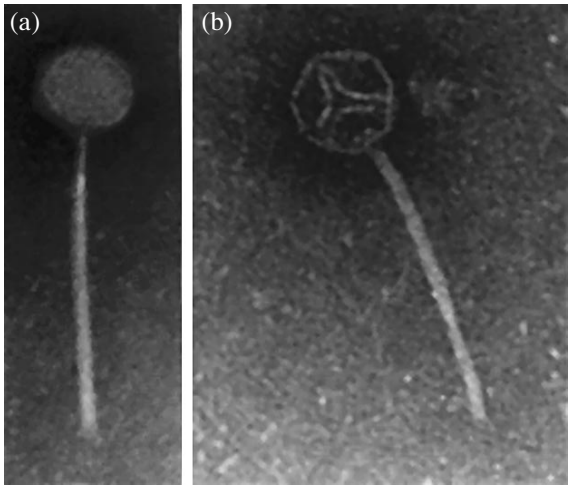

Fig. 3. Bacteriophage 1-97A particles produced by the culture of *B. thuringiensis* in the presence of the chitosan oligomer with a DP of 15. Magnification, 150000×.

The analysis of the data represented in the table shows that the chitosan oligomer inhibits bacteriophage reproduction. An increase in the phage titer in the control sample in the absence of active culture growth can be explained as follows. With an infection multiplicity of 0.1 [1, 2] that was used in this work, only one of ten culture cells is at best infected upon inoculation. The phage yield of approximately 100 particles per one cell ensures a two-order titer increase. The reproduction of daughter phage particles in the cells that were not infected upon inoculation results in a further increase in the phage titer. A decrease in the phage titer in the infected culture supernatant that is observed after 1 h of incubation with chitosan is more likely to be caused by the inactivation of unadsorbed phage particles. Considering the data on the inactivation kinetics, it can be suggested that during this time virtually all chitosan gets bound to the phage particles and, probably, to the bacterial cells. Thus, upon subsequent incubation, the inactivating effect of chitosan, including the effect on daughter phage particles, can be manifested, but slightly. Therefore, the most likely cause of the lack of phage titer increase in the test sample 3 h later is the inhibition of the production of complete phage particles by chitosan. One of the mechanisms of such an effect of chitosan may be the production of DNA-free phage particles, as shown by Fig. 3.

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