DOI 10.1007/s10517-018-4180-0
Bulletin of Experimental Biology and Medicine, Vol. 165, No. 3, July, 2018

MORPHOLOGY AND PATHOMORPHOLOGY

Influence of Specific Bacteriophage on the Level of Vesicle Formation and Morphology of Cells of *Yersinia pseudotuberculosis*

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 165, No. 3, pp. 384-388, March, 2018
Original article submitted September 14, 2017

Incubation of *Yersinia pseudotuberculosis* cells grown on a solid medium with pseudotuberculous diagnostic bacteriophage for 20 min at 37°C led to a significant decrease in the concentration of both components of the system. This effect was absent when the bacteria were grown in a fluid medium. At the same time, this incubation regimen promoted vesicle formation and typical morphological changes in bacteria grown in both surface and suspension cultures. Co-incubation of the bacteriophage with suspension of vesicles isolated from the suspension culture of *Y. pseudotuberculosis* grown at 10°C (but not 37°C) led to a decrease in plaque-forming activity of the bacteriophage.

Key Words: *Y. pseudotuberculosis; bacteriophage; vesicles*

Antibiotics remain the main treatment of infectious diseases. At the same time, antibiotic resistance acquired by pathogenic bacteria is a pressing problem. Infections caused by antibiotic-resistant bacteria are recorded in every 7th resident of Russia. The problem of antibiotic resistance is also relevant for pathogenic species of *Yersinia* genus, including *Y. pseudotuberculosis* [4].

An alternative approach to prevention and treatment of bacterial infections is the use of specific bacteriophages. Due to high specificity of action, the absence of addiction and side effects after administration, and compatibility with all drugs make bacteriophages promising therapeutic and preventive means [6].

The development of effective treatment and methods of phagotherapy requires comprehensive studies of the interaction in the two-component systems "eukaryocyte—bacterium" and "bacterium—bacteriophage", and possibly in the more complex three-component model system "eukaryocyte—bacterium—bacteriophage".

Constitutive formation of extracellular structures, the so-called vesicles (or OMVs), is typical of many gram-negative and gram-positive saprophytic and parasitic bacteria [12]. Many functions of prokaryotes are associated with the phenomenon of vesicle formation: participation in intercellular interactions, biofilm formation, microbial nutrition, export of effector proteins to host cells [2,14], transport of other, including insoluble substances: lipids, DNA, RNA, *etc*. [10,12]. Studies aimed at the development of vaccine formulations based on vesicles as the active ingredient, adjuvant or delivery system are in progress [7,8]. The importance of vesicles in protection of bacteria from antimicrobial peptides and bacteriophages [11] was shown. Such a variety of

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functions of vesicles shows their high importance in the physiology of bacteria.

Formation of vesicles was also demonstrated for one of the most dangerous bacterial pathogens, *Y. pestis* [5,9], and later for enteropathogenic species of Yersinia *Y. pseudotuberculosis* [3]. However, physiological significance of vesicle formation in bacteria of the *Yersinia* genus has not been studied.

We analyzed the effect of specific pseudotuberculous bacteriophage on vesicle formation and morphology of *Y. pseudotuberculosis* cells.

MATERIALS AND METHODS

The bacteria of *Y. pseudotuberculosis* strain 1b (Cat No. 474) and diagnostic pseudotuberculosis bacteriophage from the collection of the Russian Research Anti-Plague Institute Microbe were used in the study.

The bacterial culture was grown for 1 day at 37°C on a solid medium based on BTN-agar (Biotechnovatsia) and in a fluid medium based on the hydrochloric hydrolysate of casein. The total bacterial concentration was assessed by optical density (OD_{600}). The concentration of viable bacteria was determined by the plate method. The concentration of phage particles was determined by the Gratia method [1]. Preparations of vesicle were isolated as described previously [3], where they are marked as B antigen.

Combined incubation of bacteriophage (~ 8×10^8 PFU/ml) and microbial cells (~ 8×10^8/ml at the total concentration) was conducted at 37°C for 20 min. After the incubation, the cells were seeded by the method of serial dilution. Simultaneously, samples of bacteriophage were prepared for seeding. To this end, chloroform (2.5%) was added to the combined suspension of bacteria and phage to stop lysis and multiplication of the phage. The titer of the phage particles was determined by the Gratia method.

Fixed experimental (cells with phage) and control samples (cells without phage) by glutaric aldehyde (2.5%) were analyzed by transmission electron microscopy in negatively stained (2% uranyl acetate) and unstained form. Ultrathin sections were contrasted in a solution of uranyl acetate and lead nitrate in accordance with Reynolds [13]. The images were received with an electron microscope JEM-2100 (Jeol) at an accelerating voltage of 160 kV. Cells with vesicles were considered cells with at least 10 vesicles and/or their aggregates on their body and/or in close proximity to them. Cells with single vesicles were not taken into account.

Statistical processing of the results was conducted in accordance with standard methods with Student's *t* test. The dispersion of the mean values was a confidence interval with a significance level of 95%.

RESULTS

In preliminary studies, the ratio of the concentration of microbes and phage particles was estimated after co-incubation at 37°C (Table 1). In 20 min after mixing the two suspensions, the concentration of colony-forming bacterial cells grown on a solid medium decreased by almost 6-fold in comparison with the control (suspension of intact bacteria). At the same time, phage concentration also decreased more than 2-fold in comparison with the initial level. After using the bacteria grown in a fluid medium, at the indicated conditions of incubation, a significant change in the concentrations of microbes and bacteriophage was not observed. This indicates the presence of qualitative or quantitative differences in the interaction of bacteriophage with *Y. pseudotuberculosis*, grown in fluid and in solid media, which can be explained by the significant influence of cultivation conditions on the chemical, immunochemical composition, morphological features of the surface structures of the microbial cell, including the receptor apparatus.

Transmission electron microscopy showed that control *Y. pseudotuberculosis* culture grown on sol-

TABLE 1. Effect of Specific Bacteriophage on Vesicle Formation and Cell Size of *Y. pseudotuberculosis* (M±95%CI)

Culture medium	The study sample	Concentration of viable cells, 10^7 mb/ml	Bacteriophage concentration, 10^7 PFU/ml	Cells with vesicles, %	Cell length, μ
Solid (*n*=8)	Cells with phage	6.7±0.3	30.4±8.3	71.6±2.7	1.12±0.01
	Control 1 (cells without phage)	39.8±5.1	—	33.8±2.7	1.23±0.01
	Control 2 (phage without cell)	—	68.5±5.7	—	—
Liquid (*n*=3)	Cells with phage	28.1±11.3	47.9±21.5	67.5±8.3	1.28±0.06
	Control 1 (cells without phage)	25.8±7.1	—	17.1±6.1	1.39±0.05
	Control 2 (phage without cell)	—	62.3±16.9	—	—

Note. *n* is the number of experiments. mb — microbial bodies.

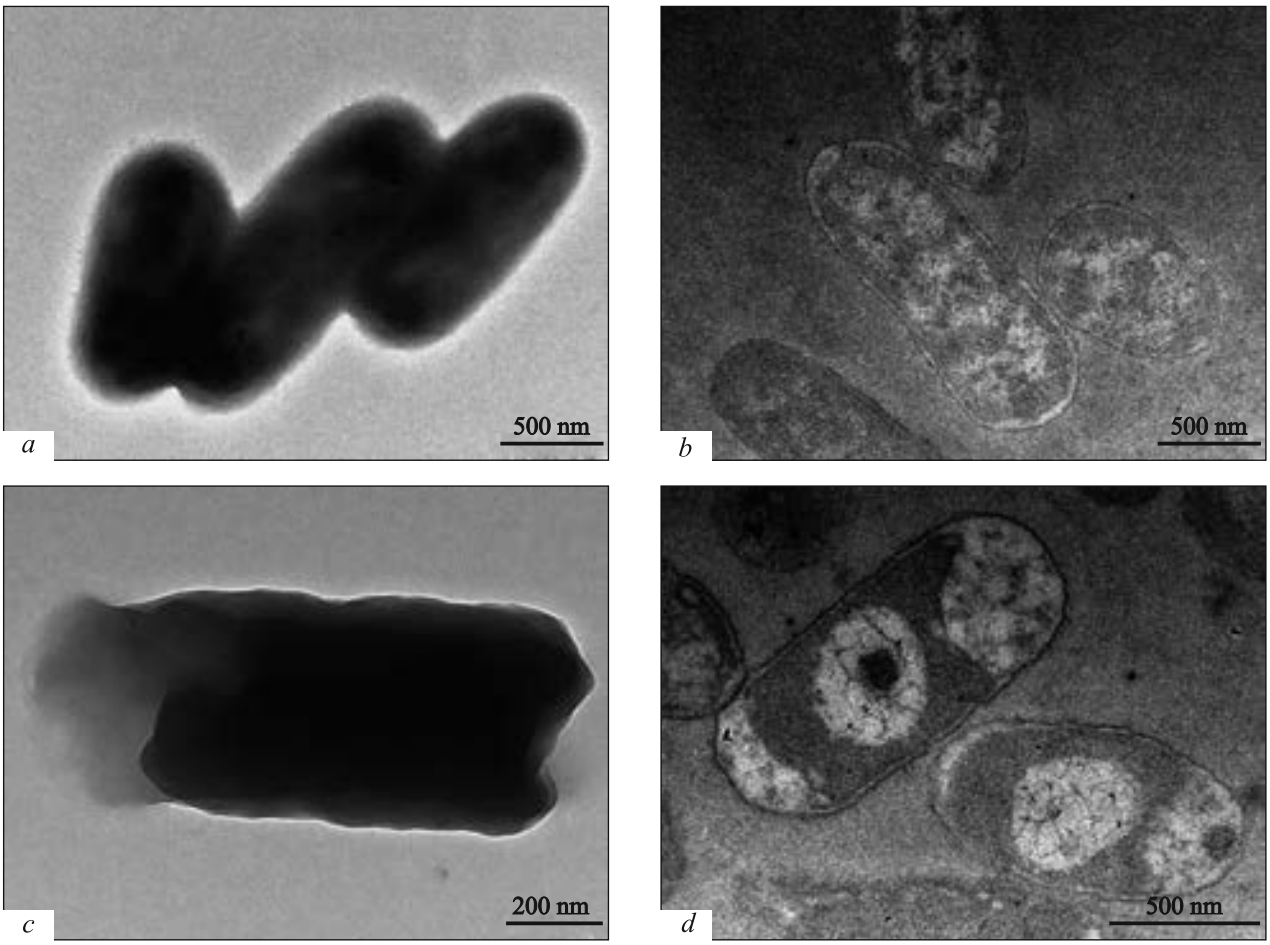

Fig. 1. *Y. pseudotuberculosis* cells: intact (*a*), treated with bacteriophage (*c*). Ultrathin sections of intact cells (*b*), treated with bacteriophage (*d*).

id medium (without bacteriophage) is presented by morphologically uniform, smooth-faced cells, often coated with a thin layer of matrix (thickness up to 50 nm). Most bacteria do not have vesicles. The cells are often collected in small dense aggregates (Fig. 1, *a*). On ultrathin sections (Fig. 1, *b*), it is seen that the cell wall does not have a pronounced sinuosity; protoplasm looks homogeneous in the all volume of cell with uniformly distributed compaction. The nucleoid is not isolated; the periplasmic space is uniform, unchanged (except for a small increase at the ends of individual cells); there are not the marked inclusions or vacuolation.

Most *Y. pseudotuberculosis* cells after incubation with specific bacteriophage had typical morphology, however, the presence of altered forms, in partixular, cells with "chopped" ends and cavity at the poles, as well as cells with a bumpy surface was demonstrated (Fig. 1, *c*). In addition, in the culture there are outlive forms (persisters). The matrix on the surface of many cells is thicker (~80 nm) than in the control, often irregular and fragmented.

Many cells showed formation of vesicle (Fig. 2). Phage particles were found mainly on the surface of microbial cells, often 2 to 3 phage particles per cell (Fig. 3) and rarely in the intercellular space. Cell ruptures and release of secondary phage particles, as well as dead bacteria in the form of empty membranes were sometimes seen.

On the ultrathin sections of the changed part of the bacteria, an increased tortuosity of the cell wall was noted, round-shaped clarification in the nucleoid region, often with a dense center. The cytoplasm of these cells was denser, the plasma membrane was clearly delineated, appreciably thickened, and somewhere detached from the cell wall, especially at the poles, with the formation of large rounded clarifications. In some cells, electron transparent granules appeared (Fig. 1, *d*). Significant shortening of the cells under the influence of a 20-min incubation with a bacteriophage is worthy of note (Table 1).

Electron microscopy of suspension cultures of *Y. pseudotuberculosis* showed that they practically did not differ by their morphology from surface bacterio-

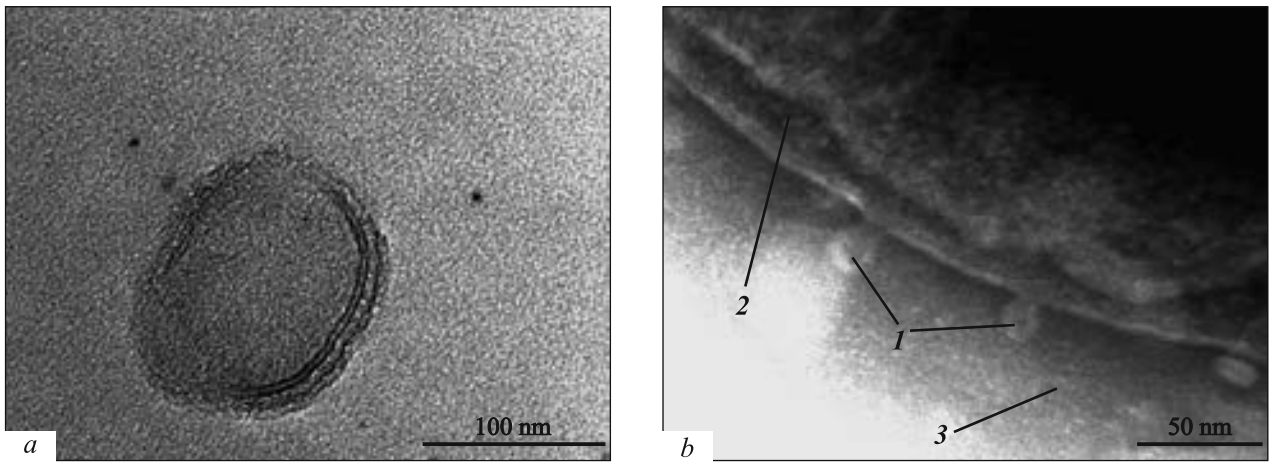

Fig. 2. Vesicle of *Y. pseudotuberculosis* (*a*), the stage of budding of the forming vesicles (*b*). *1*) Vesicles, *2*) cell body of *Y. pseudotuberculosis*, *3*) extracellular matrix.

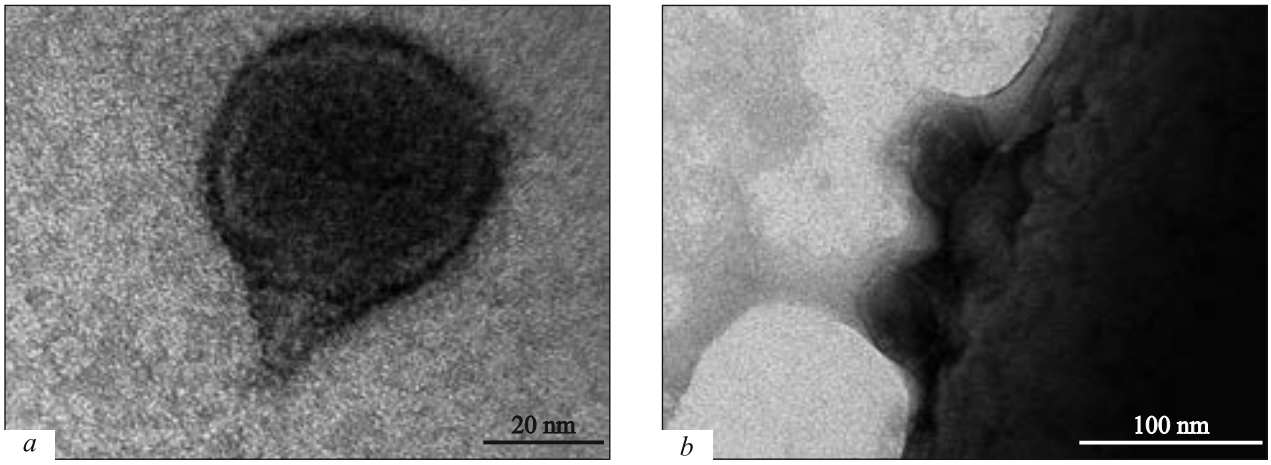

Fig. 3. Pseudotuberculous diagnostic bacteriophage (*a*), bacteriophage particles adhered to the microbial cell (*b*).

phage-treated and control cultures. However, suspension cultures consisted of cells with greater length surrounded by thicker mucous layer; they were characterized by higher content of dividing cells and lower tendency to aggregation.

The results (Table 1) indicate significant activation of vesicle formation by microbial cells after combined incubation with a suspension of specific bacteriophage; this is shown for bacterial cultures grown both by surface and in-depth method. At the same time, electron microscope revealed no direct interaction of vesicles with phage particles, which is supposed to be the mechanism of distraction of the latter from its true goal, the bacterial cell [11]. Indirectly, this assumption was confirmed in the study of influence on the plaque-forming activity of the pseudotuberculous bacteriophage of preparations of vesicles previously isolated from cultures of *Y. pseudotuberculosis*, which were grown at 10 and 37°C by a deep method. After 2 h of incubation at 37°C of a bacteriophage (~2.5×10^9 particles/ml) with vesicles (100 μg/ml), phage concentration was 1.02 ± 0.12 and $2.50\pm0.21\times10^9$, respectively, for the phage treated with "10- and 37-degree" vesicles, the concentration of untreated phage was $2.40\pm0.19\times10^9$. The difference between the concentration of the phage incubated with vesicles grown at 10°C and the concentration of the intact (control) phage was significant ($p<0.01$). The inhibition of plaque-forming activity of the phage by vesicles isolated from the "cold" culture of the pathogen is determined apparently by direct interaction with the vesicles of the phage particles that prevent their adhesion to the bacterial cell, without which it is impossible to form a negative colony in test-culture. It is likely that this effect is mediated by the action of surface-located O-side chains of LPS, whose content is high in "cold" vesicles and minimal in vesicles isolated from the culture of *Y. pseudotuberculosis* grown at 37°C [3]. It can be assumed that vesicle formation as a mechanism for protection of a pseudotuberculous microbe from specific bacterio-

phages was phylogenetically formed largely for living in an environment (temperature below 30°C) than in warm-blooded organisms. The received results confirm the known data that vesicle formation is one of the protective reactions of bacteria in response to the stressful effects of environmental factors.

The study was conducted within the framework Research and Development (State Task No. 2 0.6834.2017/BCh of the Ministry of Education and Science of the Russian Federation).

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