

Evaluation of the quality of bacterial biomass by transmission electron microscopy

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Abstract. Transmission electron microscopy can be successfully applied to quantitatively assess the quality of bacteria and microbial populations when studying the mechanism of cell inactivation at different stages of contact with disinfectants, antibiotics, biocins, including various stressful, damaging and lethal physical and chemical influences, as well as when debugging various biotechnological processes on stages of microbial cultivation, concentration and dehydration of biomass, etc.

This methodology is based on the technique of accelerated preparation of bacterial images for visualization of the fine structure of bacteria in an electron microscope and the technique of cytological analysis of the quality of bacteria in the biomass and quantitative assessment of the safety and damage of cells, diagnostics of structural disorders.

Keywords: transmission electron microscopy, bacteria, ultrathin sections, fixation of bacteria, cytological assessment of the quality of bacterial biomass, intact cells, bacterial cells with reversible damage, irreversible structural damage.

Evaluation of the effect of new disinfectants, antibiotics, biocins, physical and chemical agents on the fine structure of bacteria is carried out using the method of visualization in a transmission electron microscope of ultrathin sections, which makes it possible to reveal the features of the state of the ultrastructure of the cell both in normal conditions and after various stress and lethal effects. It should be noted that electron microscopy methods allow obtaining data both on a fairly representative sample of the population and on single cells, their structure and processes occurring in them. The method of ultrathin sections is one of the main methods of transmission electron microscopy of biological objects. With its help, the features of the fine structure of cells and supramolecular complexes are revealed, the fine structural bases of many biochemical, physiological processes are revealed; mechanisms of pathological changes in cells.

When analyzing the material, this method, by changing the magnifications, allows one to find the most subtle violations of the ultrastructure (at high magnifications), at the same time, at

low magnifications, it makes it possible to work with a fairly large sample of the microbial population.

The method consists in the fact that the biological material fixed and enclosed in a plastic mass is cut into thin sections and viewed in an electron microscope. In microbiology, the method has been most successfully applied since the last century, when, through the efforts of scientists Reiter and Kellenberger, a successful technique for fixing and dissecting bacterial cells was created, in which many of their features were taken into account. Let's list its main stages: the primary fixation of the material is carried out with a buffered solution of glutaraldehyde for 24 hours. The material is washed and embedded in agar. Additional fixation of samples is carried out for 4-16 hours in a buffered solution of osmium tetroxide (O_4), washed and additionally contrasted in a solution of uranyl acetate. Dehydration of biological material is carried out in an ethanol concentration gradient. Then the samples are impregnated in several changes of solvent and epoxy resin, for 18-20 hours each and, finally, impregnated in pure resin. At the end of the impregnation, a two-stage polymerization is carried out: 24 hours at 37°C and 48 hours at $60-70^{\circ}\text{C}$. Traditional preparation of ultrathin sections of a particular sample takes 7-12 days [1-3, etc.].

However, in order to assess the quality of microorganisms at different stages of development and testing of technologies for the production of vaccine preparations, biological products, biologically active substances and drugs of microbial origin, it is important and urgent to include express methodological approaches for visualizing the ultrastructure of bacteria and quantitative methods for assessing the quality of cells and microbial biomass at any stage of the study of the damaging effect of disinfectants, antibiotics, biocins, or at different stages of the biotechnological process.

The purpose of this work - is to develop methodological techniques for express visualization of the ultrastructure of bacteria in a transmission electron microscope and methods for assessing the quality of cells and microbial biomass at different stages of development and pilot production of microbiological preparations.

Research results and discussion

To obtain high-quality sections, the implementation of the main steps of the ultrathin section method is mandatory. Attempts to simply shorten the timing of individual stages most often ended in a deterioration in the quality of the images. Unsuccessful preparation of a biomaterial simply closes the possibility of conducting a high-quality ultrastructural analysis, therefore, any changes in the preparation technique must be justified and debugged. The traditional method of preparing ultrathin sections, albeit based on fairly accurate chemical concepts, was developed empirically and was polished in many laboratories. Taking the "working" method as a

basis, we tried to get a faster way of preparing biological material.

The developed technique of accelerated fixation and preparation of cells is based on the same basic principles of material preparation: double chemical fixation of cells, their dehydration, impregnation and filling in plastic media. But if the usual process of fixation and preparation takes about a week, then with the help of the proposed method it is completed in a day with the receipt of sufficiently high-quality images. The essence of the described technique is that in order to reduce the time for preparing bacteria for analysis, fixation and staining of cells is carried out simultaneously, excluding the stages of washing and encapsulation in agar, and the time of impregnation and polymerization of the material is reduced.

The composition of the prefix mixture in% by weight is as follows:

25% glutaraldehyde	2.0 - 2.5
Uranyl acetate	0.5 - 0.6
CaCl ₂	0.11 - 0.12
Acetate-veronal buffer pH 6.0	all the rest

According to the accelerated technique, the bacteria are initially prefixed for 5-10 minutes in the specified mixture. Then to it add 3 4% solution of osmium tetroxide in a ratio of 1:1 (volume/volume) and carry out additional fixation for 55 minutes at room temperature. The microbial suspension is centrifuged and the sediment is dehydrated in an ethanol concentration gradient: 50% 10 minutes, 70% 10 minutes, 80% 10 minutes, 90% 10 minutes, 95% 10 minutes and 100% (absolute ethanol) 3 shifts of 20 minutes. The samples are impregnated in three changes of absolute ethanol and araldite, or propylene oxide and epon in ratios of 3:1, 1:1, 1:3 at 37°C for 1.5 hours each, then transferred into pure araldite (epon) and kept in vacuum (10 ⁻⁽²⁻³⁾ torr) 1.5 hours at 37°C. The samples are embedded in fresh resin and polymerized at 90 ° C for 14 hours.

Thus, the proposed method allows for 23 hours to prepare cells for ultrastructural analysis. Further operations - making sections, additional contrasting and viewing are carried out as usual.

Let us dwell only on the most important stage of the methodology, which required maximum practicing - fixation of the material. It is the optimal fixation that allows both preserving cells in a state close to their lifetime, and protects cells from damage inevitable during dehydration, pouring, and cutting on an ultramicrotome. We used a mixture of glutaraldehyde, uranyl acetate, and osmium tetroxide for fixation. Uranyl acetate is used more as a dye to enhance image contrast. However, it was shown that rapid prefixation with a mixture of glutaraldehyde uranyl acetate makes it possible to obtain fairly good sections [4]. Perhaps this is due to the fact that 0.5% uranyl acetate gelifies a DNA solution in a few minutes, preserving the DNA structure, which has always been a preparation problem. In our own research, we have repeatedly found a positive result of a brief prefixation of biomaterials in glutaraldehyde and used this moment here. It is essential that

in the absence of laundering at the post-fixation stage, we have a mixing of fixatives. When mixed, these substances can react with each other to form components and complexes that have an additional preserving and fixing effect. It was found that the fixation of bacteria in the proposed mixture allows better visualization of the membrane structure and minimizes the extraction of nucleoid and nucleoplasm components. Note also that at the stage of impregnation of samples with resins, the best results were obtained using propylene oxide.

The study of "accelerated" sections in a transmission transmission electron microscope showed that the fine structure of the cells under study is qualitatively preserved. To test the versatility of the technique, a number of gram-positive and gram-negative microorganisms were taken: Escherichia coli strain K 12; Bacillus thuringiensis strain 52; Bacillus antracoides strain 250, etc. Fig. 1 shows micrographs of ultrathin sections of bacteria prepared by the method of accelerated fixation and preparation of cells. In the course of research, we were repeatedly convinced that the selected conditions make it possible to identify all the main elements of the ultrastructure of both vegetative and spore forms of microorganisms (fig. 1).

An important task of this study is also the introduction of a quantitative assessment of the information received. The traditional cytological description of the structure and level of preservation of microorganisms usually does not set itself the task of assessing the state of the cell population as a whole, limiting itself to visualizing individual cells and the smallest substructures. But for microbiological and biotechnological practice, a qualitative approach is clearly insufficient. In addition, a subjective moment is inevitable in assessing the state of microbial cells. Quantitative approaches to the analysis of the quality of biomass after various impacts are still lacking. Therefore, in front of us stood the task to develop an objective quantitative method for assessing the quality of biomass, which allows with a high degree of reliability to assess the level of viability of microorganisms in various biomass samples.

To find differences associated with different sensitivity of strains to a certain disinfectant or to visually represent the state of the population at different stages of processing, we could simply get images and describe them. But to describe the dynamics of the response of the microbial population to a specific drug, to compare the kinetics of alterations of different substructures, different strains, for different stress effects (antibiotics, biocins, disinfectants, etc.) was already impossible without a quantitative analysis of the material.

The proposed method of cytological analysis of the quality of cells was intended for quantitative assessment of the safety of cellular ultrastructures and diagnostics of their disorders. She proceeded from the division of the population using morphological criteria into 3 main groups based on morphological integrity.

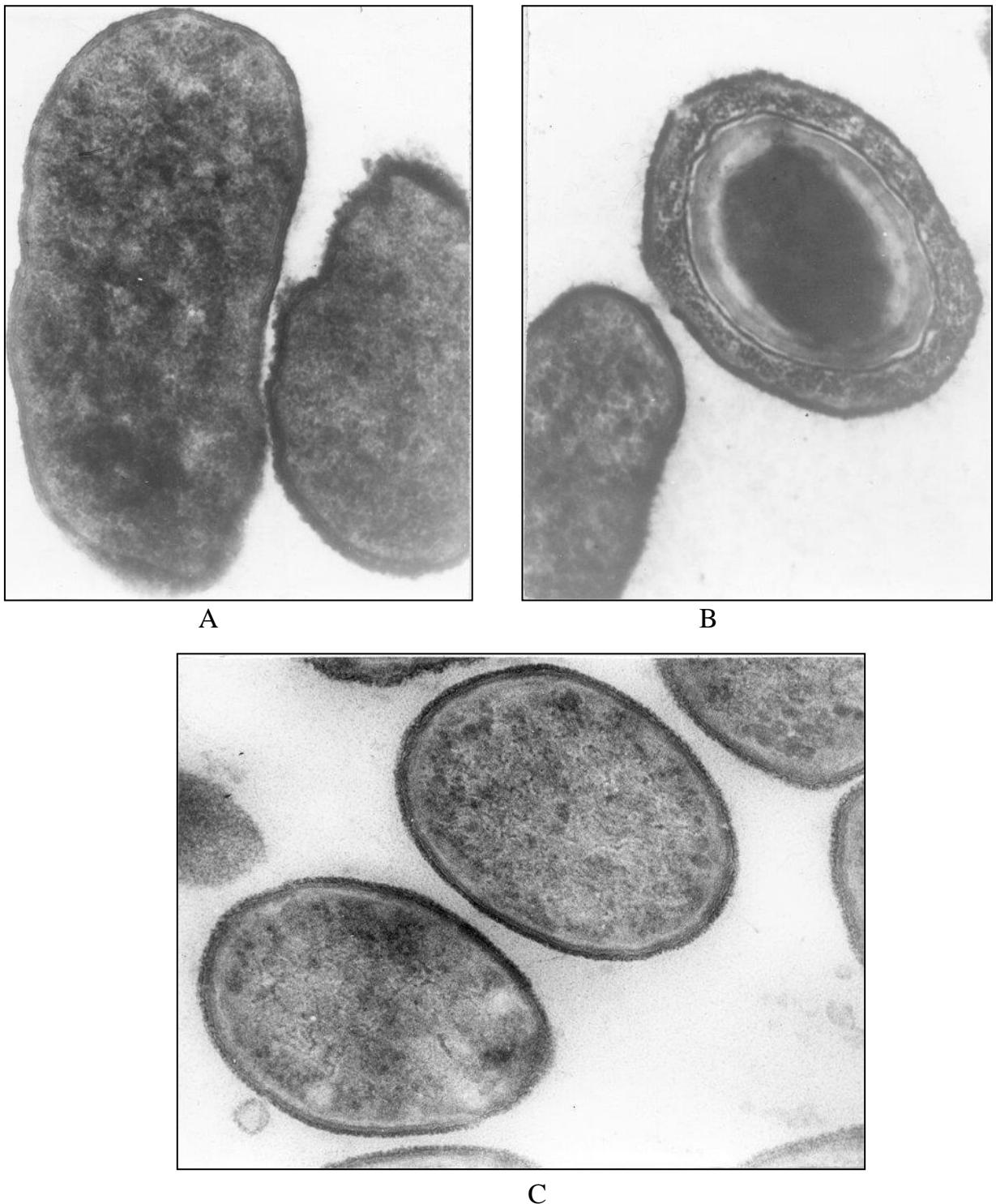


Fig. 1. Electron microscopic images of ultrathin sections of bacteria obtained using an accelerated technique for preparing bacteria and spores for examination in a transmission electron microscope. A. Electron micrograph of the fine structure of bacteria *Escherichia coli* strain K 12, magnification 85000 times. B. Micrograph of bacteria *Bacillus thuringiensis* strain 52 at the stage of sporulation, magnification 70000 times. Fine structure of bacteria *Bacillus antracoides* strain 250, an increase of 70000 times.

The method of cytological analysis is based on viewing a certain number of images of microbes, when, based on cytostructural criteria, the state of individual cells and the biomass as a whole is assessed. Practical work with ready-made sections was carried out in the following way:

ultrathin sections were viewed or photographed in a transmission electron microscope at working magnifications of 10-15 thousand times. From each sample, 15-20 random fields are photographed, which contain equatorial sections of 300-500 cells.

Hence, when visualizing the fine structure of bacteria, they are divided into three main groups of cells:

- intact or intact cells;
- cells with reversible damage;
- cells with irreversible damage, or destroyed cells.

Intact gram-negative cells are visualized with a clear, continuous, three-layer contour of the outer and cytoplasmic membranes. Intact gram-positive bacteria have one or a multi-layered homogeneous cell wall tightly adjacent to a three-layer continuous cytoplasmic membrane. For gram-negative and gram-positive bacteria in an intact state, the absence of a pronounced periplasmic space is characteristic. The cytoplasm of intact cells has a homogeneous, fine-grained structure of average electron density with a granule size of 15-30 nm, in accordance with the size of the ribosomal structures. The nucleoid has a fine fibrillar structure in the form of a compact zone of lower electron density relative to the surrounding cytoplasm. Distributed throughout the cell diffusely or centrally (fig. 2 A).

a) cells with changes in size and shape, for example, due to compression during temporary dehydration or swelling with temporary violation of permeability barriers;

b) a subgroup of plasmolyzed cells of a cell, where the main violation is the separation of the cytoplasm from the cell wall due to an increase in the osmoticity of the medium, this process can be accompanied by a partial thickening of the cytoplasm and a change in shape, but it is very important that the integrity of the membrane apparatus is fully preserved (fig. 2 C) ;

c) cells that have various violations of the cell wall in the form of ruptures of the outer membrane, or ruptures of the cell wall for gram-positive bacteria, but retaining the integrity of the cytoplasmic membrane and with an intact structure of the cytoplasm and nucleoid (fig. 2 B).

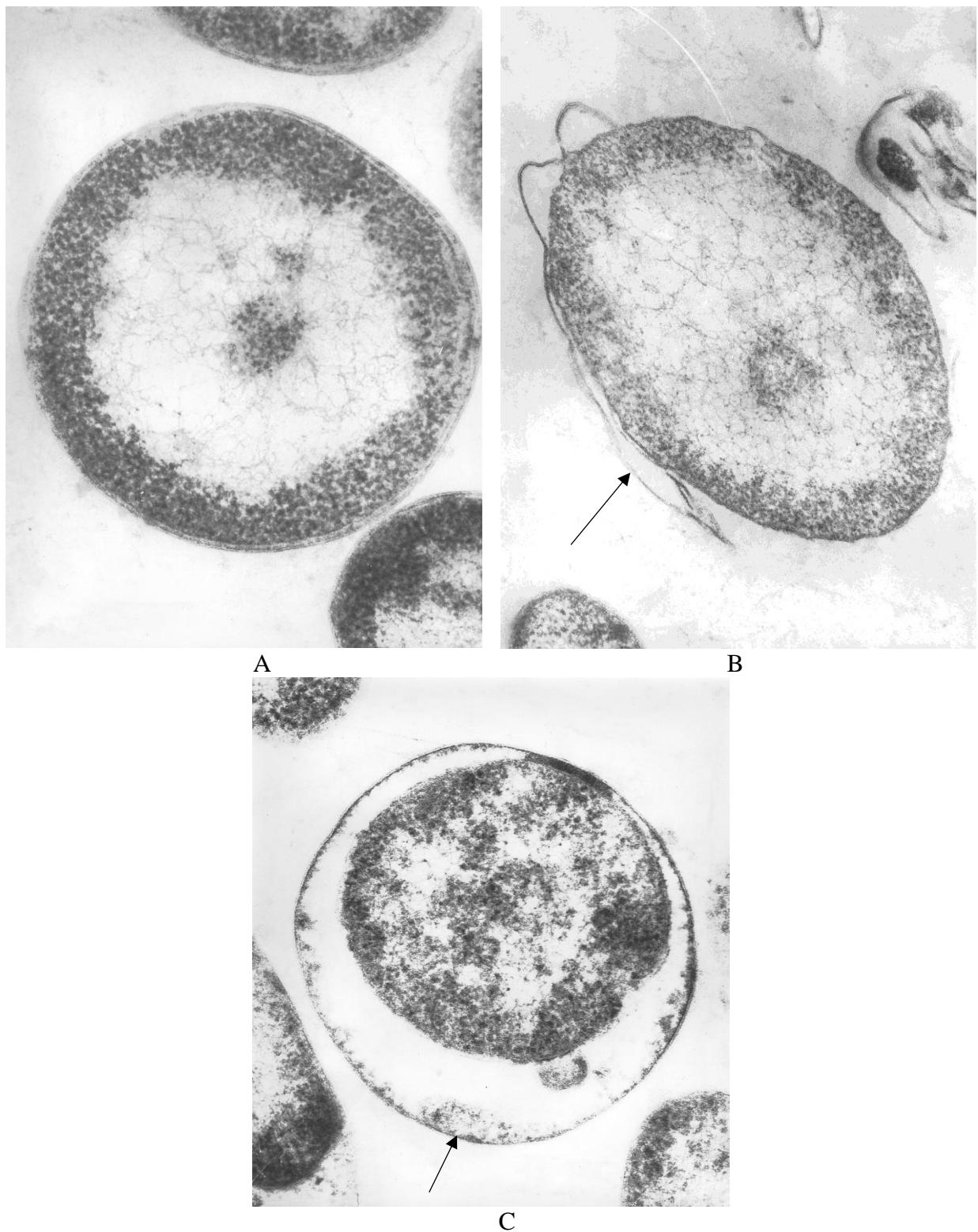


Fig. 2. Electron microscopic images of *Francisella tularensis* 15/3M bacteria with reversible ultrastructural abnormalities. A. Image of a cell with intact ultrastructure, magnification in the photo 120000 times. B. A bacterial cell with a ruptured outer membrane (arrow), magnification 100000 times. C. Image of the ultrastructure of the plasmolyzed cell (arrow), magnification 100000 times.

All these cases refer to reversible violations of the fine structure of bacterial cells. With a variety of disorders in the area of the cell wall, the integrity of the cytoplasmic membrane and the safety of the intracellular compartment are observed.

Irreversible disturbances in the vital activity of cells inevitably occur when the integrity of the cytoplasmic membrane and destructive processes in the zone of the nucleoid or cytoplasm are disturbed. Accordingly, the group of bacteria with irreversible damage includes cells that have:

- a) rupture of all layers of the cell wall, which is usually accompanied by the release of cellular contents (fig. 3 A);
- c) cells with partial destruction of the cytoplasm and nucleoid (fig. 3B);
- c) cells with complete destruction of the contents, autolyzed cells (fig. 3C).

The quantitative data are then statistically processed. The result of the analysis is to determine the percentage of intact and damaged cells in the studied population. For cells with damage, the frequency of detection of each type of damage is determined.

Conclusion. Transmission electron microscopy can be successfully applied to quantitatively assess the quality of bacteria and microbial populations when studying the mechanism of cell inactivation at different stages of contact with disinfectants, antibiotics, biocins, including various stressful, damaging and lethal physical and chemical influences, as well as when debugging various biotechnological processes on stages of microbial cultivation, concentration and dehydration of biomass, etc.

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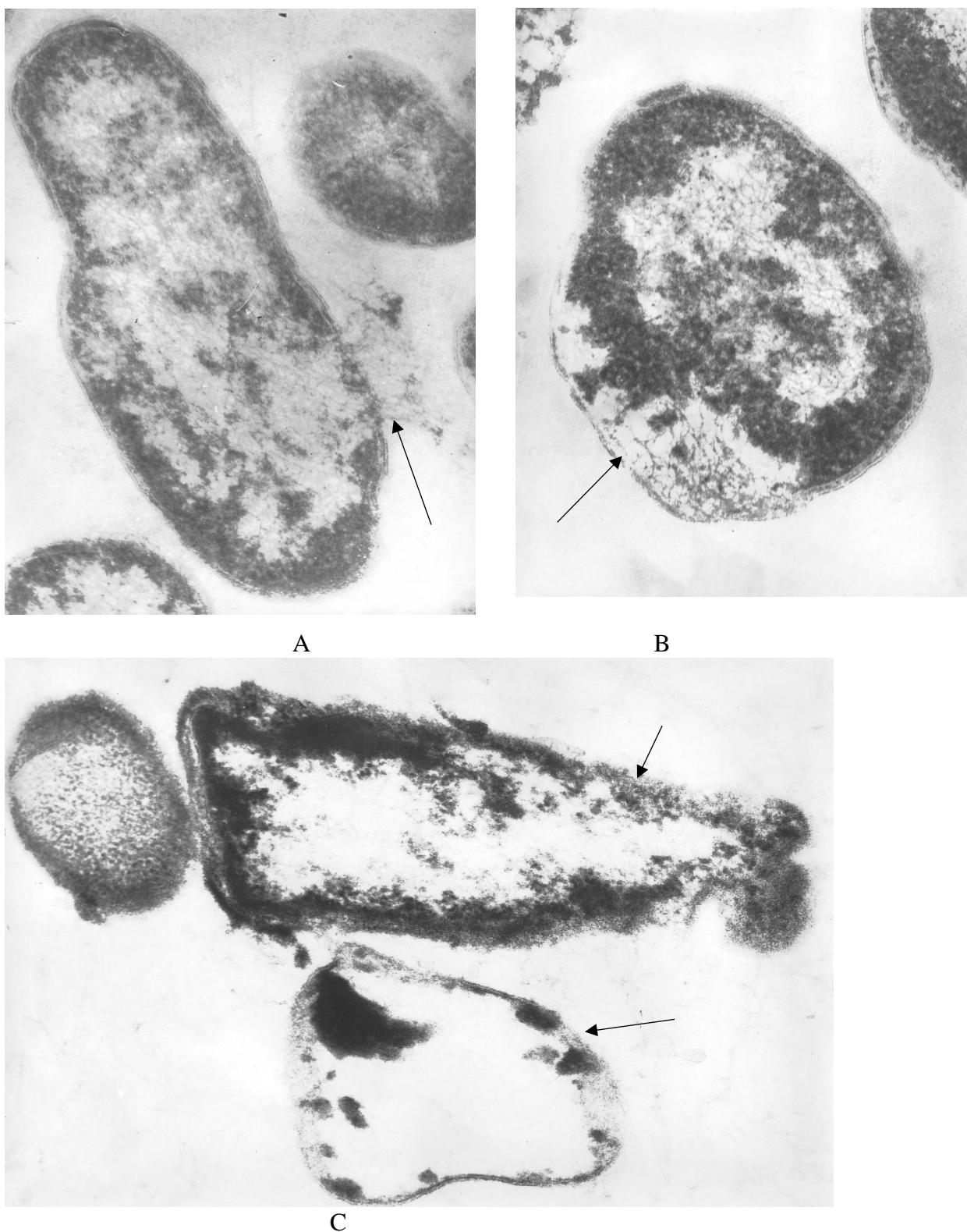


Fig. 3. Electron microscopic images of ultrathin sections of *Francisella tularensis* 15/3M bacteria with irreversible ultrastructural damage. A. Image of a cell with a rupture of the cell membrane and leakage of the cytoplasmic content (arrow), magnification in the photo 100000 times. B. Bacterial cells at the stage of destruction of the cytoplasm and nucleoid (arrow), magnification in the picture 100000 times. C. Bacterial cells at the stages of destruction and autolysis (arrow), magnification 80000 times.

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