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EXPERIMENTAL ARTICLES

Electron Microscopy of the Surfaces of Bacillus Spores

T. A. Smirnova[a, 1], M. V. Zubasheva[a], N. V. Shevlyagina[a], M. A. Nikolaenko[b], and R. R. Azizbekyan[b]

[a] *Gamaleya Research Institute of Epidemiology and Microbiology, ul. Gamaleya 18, Moscow, 123098 Russia*

[b] *State Research Institute of Genetics and Selection of Industrial Microorganisms, Pervyi Dorozhny proezd 1, Moscow, 117545 Russia*

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Abstract—The surface structures of the spores of *Bacillus cereus, Bacillus thuringiensis*, and *Brevibacillus laterosporus* were studied by transmission and scanning electron microscopy. Platinum deposition and negative staining with uranyl acetate revealed appendages and exosporium in *B. thuringiensis* and *B. cereus*. The exosporium structure was visualized by negative staining and ultrathin sectioning. For staining the exosporium polysaccharide, Alcian blue was used during fixation. The results obtained show the differences in structural organization of appendages and exosporium in different strains. Canoe-shaped inclusions were revealed in all *Br. laterosporus* strains, while strain IGM16-92 had a fibrillar capsule as well. Electron microscopy using a dual beam scanning electron microscope Quanta 200 3D provided the information of the spore surface relief without sample treatment (fixation and dehydration). The spores of *Br. laterosporus* strains had folded surface, unlike the smooth surface of *B. cereus* and *B. thuringiensis* spores. The diversity of external spore structures was shown within a species, which may be used for detection of bacteria at the strain level. Optimized procedures for visualization of spore surface by different electron microscopic techniques were discussed.

Keywords: transmission and scanning electron microscopy, bacilli *B. cereus, B. thuringiensis*, and *Br. laterosporus,* spore appendages, exosporium, canoe-shaped inclusion

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Bacterial spores are presently a subject of intense study, including cytological investigation, which makes it possible to establish connections between the biological and physicochemical properties of the spores and the features of their specific surface structures. Spores are the best studied of the dormant forms (DF), which are used for the propagation and preservation of a species [1]. Wide occurrence of spores (in water, air, soil, etc.) and preservation of their viability under various conditions, including extreme ones, depend on the specialized spore structures. Species propagation by spores, which have no locomotory organelles and are therefore incapable of independent movement, occurs by passive transfer with the flow of air or water, as well as by transfer by insects and animals. Due to their capacity for adhesion, the spores may be attached to various objects and fall into various ecological niches. Outer structures of bacterial spores (appendages, exosporium, and spore envelope) are involved in this process. Morphological organization of the surface structures of bacterial spores determines their behavior in the environment and attracts much attention. In spite of its role in bacterial colonization of new suitable areas, this is as yet a poorly studied aspect of spore cytology.

The present work deals with morphological characterization of the cell surface. Spores of bacteria of the *Bacillus cereus–B. thuringiensis* group were chosen for the morphological analysis of their surface structures by a variety of electron microscopic techniques. Spore-forming aerobes of this group are widespread soil bacteria, mostly saprotrophic [2], although toxicogenic strains occur. *B. thuringiensis*, the species related to *B. cereus*, is the best studied entomopathogenic bacterium, which is used for production of efficient, environmentally safe preparations against insect pests. In some cases, insecticidal activity of *B. thuringiensis* results from combined action of spores and crystalline proteinaceous toxins.

The investigated spores of *B. cereus* and *B. thuringiensis* share some morphological characteristics. They have an external sheath (exosporium) and appendages determining their hydrophobicity and capacity for adhesion [3, 4]. Spore appendages have been previously described for clostridial spores [5]. Spore appendages of bacilli are, however, different from such structures in clostridia. In *B. cereus*, the size

[1] Corresponding author; e-mail: smiryu@mail.ru

and number of spore appendages depends on the strain. Spore appendages of *B. cereus*, as well as its exosporium, were shown to participate in adhesion to stainless steel [6, 7]. We have previously shown that adhesive activity of *B. thuringiensis* spores to plant leaves and erythrocytes depended on the presence of spore appendages [8, 9].

Apart from *B. thuringiensis*, less studied entomopathogenic spore-forming bacteria *Brevibacillus laterosporus* (*Br. laterosporus*) attracted attention recently. We found that some *Br. laterosporus* strains produced crystals with antimosquito activity [10–12], which may be used for development of alternative antimosquito preparations. Some *Br. laterosporus* strains produce biologically active compounds, antibiotics, fungicidal and algicidal factors, etc. [13–16]. Application of *Br. laterosporus* strains as probiotic spore preparations was discussed [17]. Thus, the investigated species *B. thuringiensis* and *Br. laterosporus*, while similar in their capacity for production of crystals, differed in spore morphology. The presence of crystals in environmental isolates hampers species identification during screening of entomopathogenic bacteria. Their determination, including the one based on the structural features of the spores, is therefore desirable. Unlike *B. thuringiensis*, behavior of *Br. laterosporus* in the environment is poorly studied and requires further investigation.

Electron microscopy is widely used for investigation of spore ultrastructure. In our previous works, spore structures of bacilli were described and significant strain variations were noted. Thus, large-scale analysis of the organization of bacterial spores using a number of strains will improve our understanding of the structure of spore surfaces. The technique for electron microscopy of spores is of importance. Results obtained using a dual beam scanning electron microscope Quanta 200 3D are of special interest. Comparative analysis of various techniques will make it possible to carry out the optimal choice adequate to the task.

The goal of the present work was to visualize the surface structures of the spores of various strains of bacilli by transmission and scanning electron microscopy.

MATERIALS AND METHODS

Bacterial strains. The following strains were used in the present work: *Bacillus thuringiensis* subsp. *thuringiensis* 98, *B. thuringiensis* subsp. *kurstaki* HI, *B. thuringiensis* subsp. *galleriae* 69-6, *B. thuringiensis* subsp. *israelensis* 1-5, *B. cereus* Gp-7, *B. cereus* 569, and *Brevibacillus laterosporus* (formerly *Bacillus laterosporus* [18]) BL IGM16-92 and BL52 from the collection of the State Research Institute of Genetics and Selection of Industrial Microorganisms. *Br. laterosporus* strain Lat 006 was provided by Dr. Lekadet (collection of entomopathogenic bacteria, Institute Pasteur, Paris, France).

Cultivation conditions. The strains were grown on LB agar containing the following (g/L): tryptone, 10; yeast extract, 5; NaCl, 10. The cultivation was carried out at 30°C for 48–96 h (until completion of the sporulation, which was confirmed by light microscopy).

Spore preparations. The spores were washed off with sterile distilled water, washed twice (7300 *g*, 5 min), resuspended in sterile distilled water, and stored at 4°C.

Spore hydrophobicity was determined by adhesion on hexadecane [3].

Transmission electron microscopy. Spore preparations were applied to the grids with carbon-coated formvar films. The preparations were shadowed with platinum or contrasted with 1% aqueous uranyl acetate.

The samples for ultrathin sectioning were treated as described previously [19]. The sections were made on an LKB 3 ultramicrotome (Sweden). The sections were contrasted with ethanol solutions of uranyl acetate and lead citrate. Staining with Alcian blue was carried out during fixation according to [20]. The preparations were examined under a JEM 100 B electron microscope (Jeol, Japan) at 80 kV.

Scanning electron microscopy. Spore surface was studied under a dual beam scanning electron microscope Quanta 200 3D (FEI Company, United States). The spores were not fixed, dehydrated, or dried. In some cases they were sputter coated with gold (5 nm). Spore samples were placed on a silicon stub, which was mounted on an aluminum stage with conductive adhesive tape. The samples were examined under low and high vacuum at 5–30 kV.

RESULTS AND DISCUSSION

Numerous studies showed that the morphological diversity of spores was combined with the heterogeneity of their physicochemical and biological characteristics. In the present work, the spores of *B. thuringiensis* subspecies *thuringiensis, galleriae, kurstaki*, and *israelensis*, of *Br. laterosporus* strains BL IGM16-92, BL 52, and Lat 006, as well as nonpathogenic strains *B. cereus* Gp-7 and 569 were used in the present work [21, 22].

Spore hydrophobicity. Hydrophobicity of the spores, which depends on the presence of the exosporium and appendages, is one of their important physicochemical characteristics. Assessment of hydrophobicity of the spores of *B. cereus* (strains Gp-7 and 569) and of *Br. laterosporus* strain BL 52 by adhesion to hexadecane revealed that hydrophobicity indices for

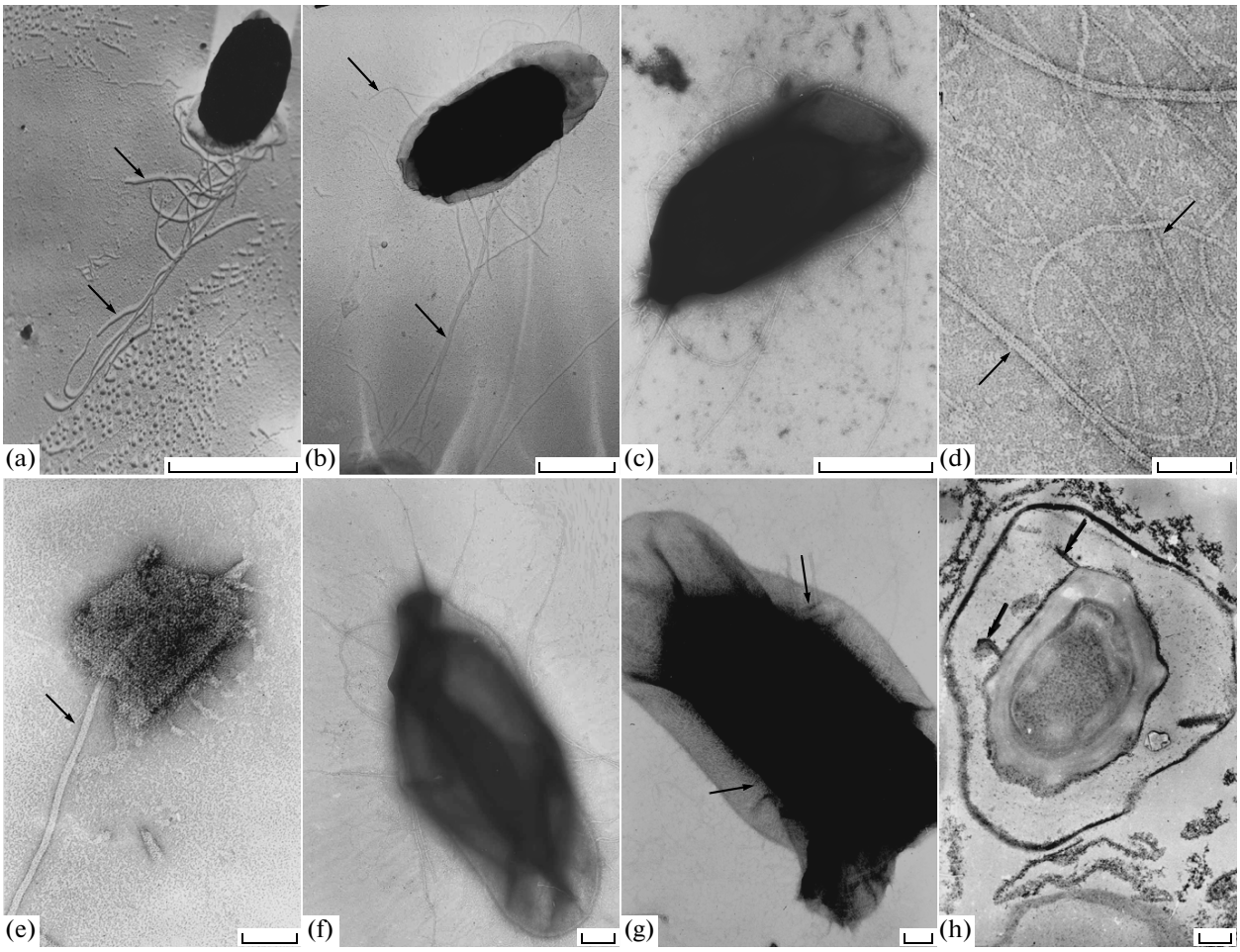

Fig. 1. Spore appendages in *B. thuringiensis* and *B. cereus*: appendages on *B. thuringiensis* subsp. *galleriae* spores (arrows), platinum coating, scale bar = 1 μm (a); appendages of *B. thuringiensis* subsp. *israelensis* spores (arrows), platinum coating, scale bar = 1 μm (b); appendages of *B. thuringiensis* subsp. *galleriae* spores, negative staining, scale bar = 1 μm (c); thin tubular and filamentous appendages on *B. thuringiensis* subsp. *galleriae* spores (arrows), negative staining, scale bar = 100 nm (d); exosporium fragment with a tubular appendage, *B. thuringiensis* subsp. *galleriae*, negative staining, scale bar = 100 nm (e); appendages of *B. cereus* 569 spores, negative staining, scale bar = 100 nm (f); appendages inside the exosporium of *B. cereus* GP-7 spores (arrows), negative staining, scale bar = 100 nm (g); appendages associated with the spore envelope in *B. cereus* GP-7 (arrows), ultrathin section, scale bar = 100 nm (h).

strains Gp-7 and 569 were 25 and 54%, respectively. The relatively low hydrophobicity of Gp-7 spores resulted from the absence of the surface appendages increasing hydrophobicity. After mixing with hexadecane, the spores of strain BL 52 remained in the water phase. This may be the reason the spores of *Br. laterosporus*, which have neither exosporium nor appendages, occur at the sites inhabited by mosquito larvae.

Transmission electron microscopy. Spore surfaces were studied on negatively stained and shadowed preparations. Coating revealed projections of various morphology at the surface of bacterial spores (Figs. 1a, 1b). In *B. thuringiensis* subsp *galleriae*, long flexible projections (much longer than the spore size) were observed. In *B. thuringiensis* subsp. *israelensis*, apart from such structures, straighter thin structures contacting with each other were found. Appendages of the latter type in this strain may form bundles [9]. Negative staining with uranyl acetate both visualized the spore appendages and revealed their ultrastructure (Figs. 1c, 1d). Tubular and filamentous appendages characteristic of *B. thuringiensis* subsp. *galleriae* are shown on Fig. 1d. The length of tubular appendages was 4 μm, their diameter was 6–10 nm. Staining with uranyl acetate revealed internal channels in the tubular appendages (Fig. 1d). The appendages had peritrichous localization, were closely associated with the exosporium, and were observed only at its outer side (Fig. 1c). Sonication of the spores resulted in emergence of exosporium

fragments with attached tubular appendages (Fig. 1e). A flagellar fragment seen of Fig. 1e is much thicker than the tubular appendages.

Similar long appendages were observed in *B. cereus* 569 spores (Fig. 1f). Strain *B. cereus* Gp-7 was found to possess unusual appendages. They filled the exosporial space, as was revealed by negative staining (Fig. 1g). Their association with the spore envelope was revealed by ultrathin sectioning (Fig. 1h). They were not observed at the external surface of the spore.

Spore appendages involved in adhesion are functionally similar to the fimbria of gram-negative bacteria, such as rhizobacteria. Various rhizobacteria participate in plant colonization, the process involving their surface structures, including fimbria. *B. cereus* and *B. thuringiensis* were also revealed in the rhizosphere [23]. Spore appendages of bacilli may participate in the interaction with plants. The rhizosphere is a reservoir of microorganisms interacting both with plants and with other hosts. Due to their spore structures, soil bacteria *B. cereus* and *B. thuringiensis* are able to adapt to new conditions and to grow in the intestine of insects and mammals [24].

Because *B. cereus* may cause food poisoning in the case of contaminated foodstuffs and food manufacturing equipment, resistance of *B. cereus* spores and techniques for their decontamination attract much attention.

In *B. cereus* and *B. thuringiensis*, the exosporium forms the sheath surrounding the mature spore and acting as an additional barrier protecting it from unfavorable environmental factors and preventing the biocides from penetration into the germinating spore. The exosporium contains proteins and glycoproteins [7]. Details of the exosporium structure were not revealed on metal-coated preparations. Negative staining revealed the structure of the exosporium. In *B. thuringiensis* subsp. *galleriae* the exosporium consisted of a basal layer with crystalline packing of the subunits and the outer layer contained hair-like projections (Fig. 2a). Ultrathin sections also revealed the exosporium structure. Both its basal layer and the outer layer of short filaments were visible. In some *B. thuringiensis* strains, the exosporium of a complex structure was revealed, double or flexible, folded (Figs. 2b, 2c). In *B. thuringiensis* subsp. *thuringiensis*, the exosporium structure was more simple (Fig. 2d). This microphotograph shows the contact between the exosporium and the crystal, which probably holds them together for some time after formation of the spore and the crystal was completed. In *B. thuringiensis* subsp. *israelensis* an open exosporium was found (Fig. 2e). Evidence exists suggesting that, in the case of an open exosporium, large particles, including the phages, may penetrate the exosporal space [25]. The open exosporium may play a role in spore germination, providing for the contact between the spore content and the environment.

Ultrathin sections of *B. cereus* 569 revealed the exosporium visible as a clear line on the sections (Fig. 2f). This type of the exosporium did not exhibit the fringe of short filaments, which was well pronounced in some *B. thuringiensis* strains. Much attention has been recently paid to the structure of the exosporium, which is responsible for the interaction between spores and external objects. The role of glycoproteins of the exosporium in its surface and adhesive properties was studied. The BclA glycoprotein was shown to be the major component of the filamentous fringe of the exosporium in *B. cereus* ATCC14579. Some data indicate that the polysaccharides at the outer side of *B. cereus* spores may be revealed by electron microscopy of the preparations stained with Ruthenium red and Alcian blue [26]. Staining *B. cereus* 569 spores with Alcian blue resulted in improved contrast of the external spore surface, indicating the presence of acidic polysaccharides with affinity to Alcian blue (Fig. 2h).

Our findings confirm the diversity of the surface structures in the spores of various *B. cereus* and *B. thuringiensis* strains. For instance, differences in both the structure and the hydrophobic properties between the spores of *B. cereus* 569 and Gp-7 were revealed (see Spore hydrophobicity section). These results are applicable to the spores of *B. cereus* and *B. thuringiensis*, which possess the exosporium and appendages. These structures are not, however, present in all spores. For example, *Br. laterosporus* spores have no exosporium. The spores of *Br. laterosporus* are characterized by a canoe-shaped inclusion, which is formed in the sporangium and is attached to the mature spore. Apart from the spore envelope, *Br. laterosporus* spores have numerous repeated layers similar to the envelope and forming the canoe. We investigated *Br. laterosporus* by transmission and scanning electron microscopy (Figs. 3, 4). In the course of sporangium lysis, the canoe-shaped inclusion was released together with the spore. Negative staining revealed uneven surfaces of the spore and canoe (Fig. 3a). Lamellar structure of the canoe was visible on ultrathin sections. The canoe of strain Lat 006 was significantly larger than that of strain BL IGM16-92.

Spores of *Br. laterosporus* strain IGM16-92 possessed a broad fringe of thin fibrils, which was not revealed by negative staining (Fig. 3b). This layer covered the spore evenly, forming a capsule-like structure. By the time of its emergence from the sporangium, the spore becomes completely isolated from the environment by a kind of a fibrillar capsule. The capsule of *Br. laterosporus*, similar to the exosporium in other bacilli species, is probably responsible for additional resistance of the spore. Prolonged storage (3 months at 4°C) resulted in a partial loss of the capsule, which

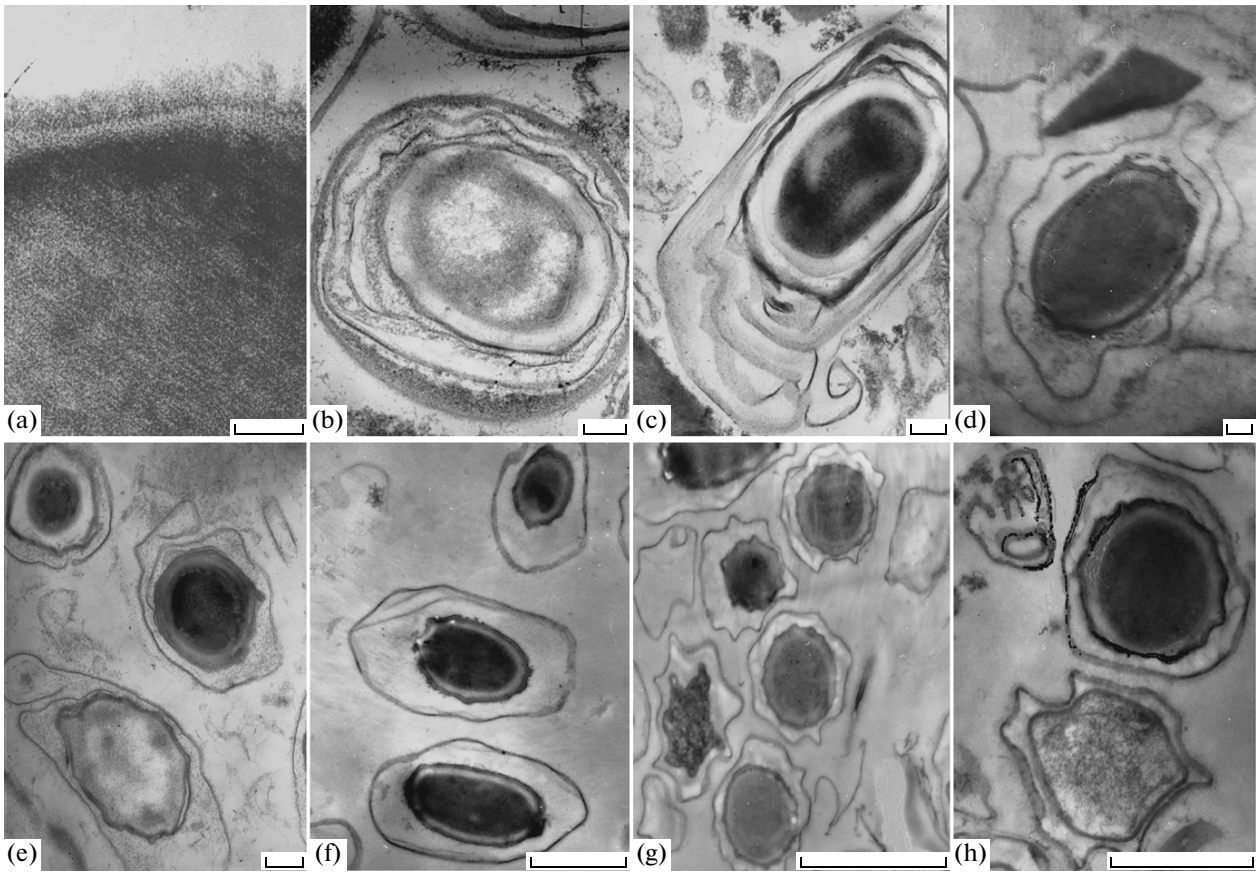

Fig. 2. Exosporium in *B. thuringiensis* and *B. cereus* spores: fragment of *B. thuringiensis* subsp. *galleriae* exosporium, negative staining, scale bar = 100 nm (a); complex exosporium in *B. thuringiensis* subsp. *galleriae*, ultrathin section, scale bar = 100 nm (b); complex exosporium in *B. thuringiensis* subsp. *kurstaki*, ultrathin section, scale bar = 100 nm (c); spore and crystal in *B. thuringiensis* subsp. *thuringiensis*, ultrathin section, scale bar = 100 nm (d); open exosporium in *B. thuringiensis* subsp. *israelensis*, ultrathin section, scale bar = 100 nm (e); *B. cereus* 569 spore, ultrathin section, scale bar = 1 μm (f); *B. cereus* 569 spores unstained with Alcian blue, ultrathin section, scale bar = 1 μm (g); *B. cereus* 569 spores stained with Alcian blue, ultrathin section, scale bar = 1 μm (h).

could be hardly visualized without specific staining. Staining with Alcian blue revealed thin filaments, which enhanced their contrast and indicated the polysaccharide nature of external spore structures. Similar to *B. cereus* exosporium, the external surface of *Br. laterosporus* spores probably contains glycoproteins, which are involved in the interaction with the environment. Thus, investigation of the spores of different *Br. laterosporus* strains revealed their morphological differences, especially in the structure of canoe-shaped inclusions.

Scanning electron microscopy. Scanning electron microscopy is an especially attractive approach to investigation of spore surfaces. Conventional scanning electron microscopy requires fixation and dehydration of the samples (dehydration in alcohol and critical point drying). This treatment affects the structure of biological objects. The dual beam scanning electron microscope Quanta 200 3D makes it possible to study the specimens without preliminary treatment. The main goal of this part of the work was to assess the possible application of the Quanta 200 3D microscope for visualization of the spore structures. We have previously determined the conditions for the sample preparation [27]. The results were compared with those obtained by transmission electron microscopy. Investigation of *B. thuringiensis* and *B. cereus* preparations revealed that the spores had a relatively smooth surface and were covered with exosporium (Fig. 4a). Two crystal-bearing *Br. laterosporus* strains, Lat 006 and IGM16-92, were also analyzed. Their spores have a characteristic feature, a canoe-shaped inclusion attached to the spore. The size of the canoe was 0.5–0.6 μm and 0.25–0.4 μm for strains Lat 006 and IGM16-92, respectively. SEM photographs provided reasonably distinct images of the canoe (Figs. 4c, 4d). The surface of *Br. laterosporus* spore looked folded.

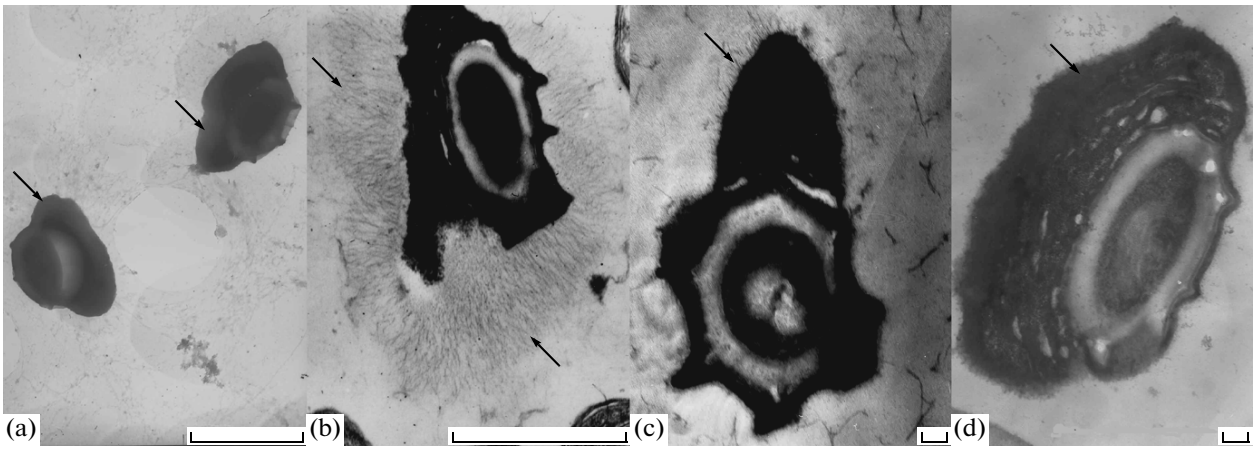

Fig. 3. *Br. laterosporus*, spores with canoes: BL IGM16-92 spores with canoes (arrow), folded envelope, and attached crystal, negative staining, scale bar = 1 μm (a); BL IGM16-92 spore with a canoe, surrounded by a fibrillar capsule (arrow), ultrathin section, scale bar = 1 μm (b); BL IGM16-92 spore with thin filaments (arrow), stained with Alcian blue, ultrathin section, scale bar = 100 nm (c); *Br. laterosporus* LAT 006, spore with a canoe (arrow), ultrathin section, scale bar = 100 nm (d).

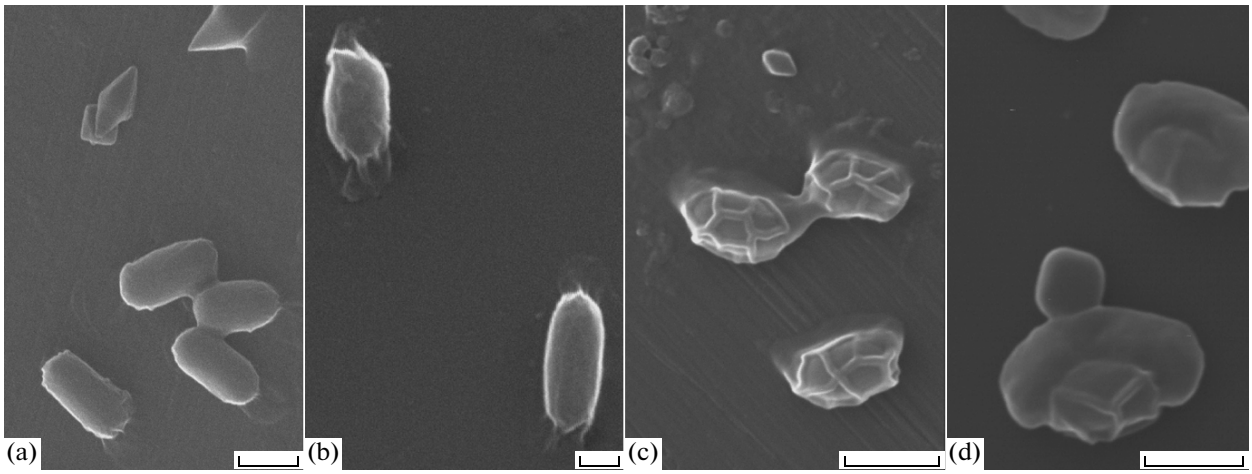

Fig. 4. Scanning electron microscopy: *B. thuringiensis* subsp. *kurstaki*, crystals visible, scale bar = 1 μm (a); *B. cereus* 569, scale bar = 1 μm (b); BL IGM16-92 spore with folded envelope and canoe, a crystal visible, scale bar = 1 μm (c); BL LAT 006, spore with folded envelope and canoe, a crystal associated with the spore visible, scale bar = 1 μm (d).

The folds were especially noticeable in the case of scanning electron microscopy of uncoated preparations. According to the results of scanning electron microscopy of the bacterial spores studied in the present work, they differed in their surface structure. These results show that the information for detailed morphological characterization of the spores may be obtained without special sample treatment (fixing and dehydration). The surface relief was especially visible. It should be noted, however, that scanning electron microscopy failed to visualize spore appendages of *B. cereus* and the capsule of *Br. laterosporus*.

Analysis of the results obtained by transmission and scanning electron microscopy (table) shows that a combination of these methods is able to reveal the features of structural organization of the spore surface correlating with their physical and biological properties. Our research revealed some previously unknown structural features of the spores of two *B. cereus* strains and specified the particulars of spore structure in *B. thuringiensis* and *Br. laterosporus*. These results improve our knowledge of the structure of bacterial spores, which is important for the understanding of survival and propagation of these bacteria in the environment.

Visualization of spore structures by different methods

Methods	Spores		
	B. thuringiensis	*B. cereus*	*Br. laterosporus*
Transmission electron microscopy, shadowing	Exosporium, appendages	Exosporium, appendages	N.d.*
Transmission electron microscopy, negative staining	Exosporium, appendages	Exosporium, appendages	Canoe-shaped inclusion
Transmission electron microscopy, ultrathin sections	Exosporium	Exosporium	Canoe-shaped inclusion, fibrillar capsule
Scanning electron microscopy	Smooth surface relief, exosporium	Smooth surface relief, exosporium	Folded surface relief, canoe-shaped inclusion

* N.d. stands for no data.

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