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EXPERIMENTAL ARTICLES

The Architectonics of Colonies of *Bacillus subtilis* 2335

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Abstract—Colonies grown from vegetative *Bacillus subtilis* 2335 cells had a standard structure, with bacillar cells occupying the whole colony volume. At the same time, the colonies of this bacterium grown from germinated spores had an abnormal structure characterized by the location of cells in a surface layer 100–200 μm thick at the colony boundary with the air. The glycocalyx of the colonies grown from spores was characterized by a wetting angle θ_e of 120°–160°, whereas that of the colonies grown from vegetative cells had an angle θ_e as low as 5°–30°. It is suggested that spores and vegetative cells follow different strategies of substrate colonization and that the architectonics of bacterial colonies is determined by the physicochemical properties of the glycocalyx.

Key words: electron microscopy, *Bacillus subtilis*, spores, vegetative cells, glycocalyx, architectonics of bacterial colonies.

It is generally believed that microbial cells in colonies are uniformly distributed over the colony volume. This belief descends from the conception that the growth of bacteria on solid nutrient media is controlled by the diffusion of nutrients from the medium to the cell and excreted metabolites in the opposite direction [1, 2]. This conception implies that good bacterial growth is provided by the location of cells directly on the surface of nutrient agar or by close packing of cells in colonies, which minimizes the diffusion lengths of nutrients and excreted metabolites.

In the present paper, we report microscopic evidence that the architectonics of *Bacillus subtilis* colonies does not agree with this conception and discuss the possible mechanisms of formation of such colonies.

MATERIALS AND METHODS

Strain and growth conditions. The *Bacillus subtilis* strain 2335 (VKPM B-4759) used in the present work was obtained from the All-Russia Collection of Industrial Microorganisms (VKPM). The strain was described in detail by Krylova *et al.* [3]. The strain was grown at 30°C on an agar M9 medium containing 20 g/l agar, 5 g/l peptone and 2 mg/ml glucose [4] for 5–6 days. The inoculum was either vegetative cells from the 5th subculture or spores obtained by keeping vegetative cells from the 4th subculture for 2 weeks at 4°C.

Microscopy. Specimens for microscopic analysis were prepared as described earlier [5]. Semithin and ultrathin vertical sections of colonies were cut using a Reichert UM-03 ultramicrotome (Austria). To study the surface of colonies by scanning microscopy, the colonies embedded in a resin were bared using a TM60 cutter (Austria), polished with a diamond paste, and etched with 1 M NaOH. The section was mounted onto a specimen support and coated with aluminum in a JEE-4C vacuum evaporator (Japan). Scanning and scanning transmission electron microscopic images were processed on a personal computer, into which the images were transmitted from the EM-ASID-4 scanning attachment of a JEM-100C electron microscope with the aid of a home-made analog-to-digital converter. Light microscopic images were analyzed with the aid of a Carl Zeiss image processing system and a DM 23 binocular (Czech Republic) connected through a Logitech QuickCam Home video camera to a personal computer.

Terminology. Colonies were studied taking into account their spatial position during growth. It should be noted that colonies are commonly depicted as heaps of cells grown on the surface of an agar medium. Actually, however, to avoid the dripping of condensed water onto the agar surface, colonies are grown in inverted petri dishes, so that the colonies are hanging from the lower surface of the agar plate. As a result, the top of a growing colony of a microorganism not penetrating into agar media (such as *B. subtilis*) is the contact plane with the agar. The bottom of such a colony is the part remote from the agar (Fig. 1). Bearing this in mind, it would be more correct to call smooth domed colonies cup-shaped colonies (Fig. 1a). In the case of wrinkled

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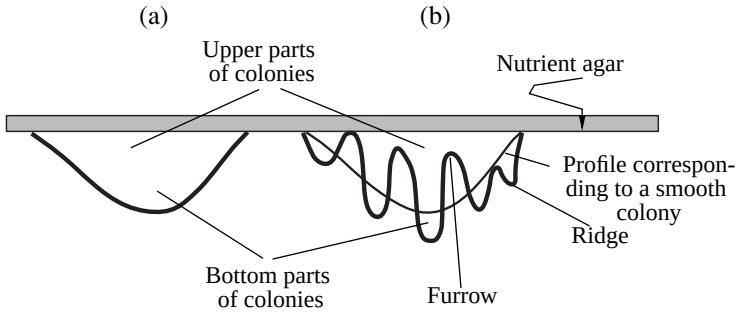

Fig. 1. Schematic representation of (a) cup-shaped and (b) wrinkled *B. subtilis* 2335 colonies grown in an inverted petri dish.

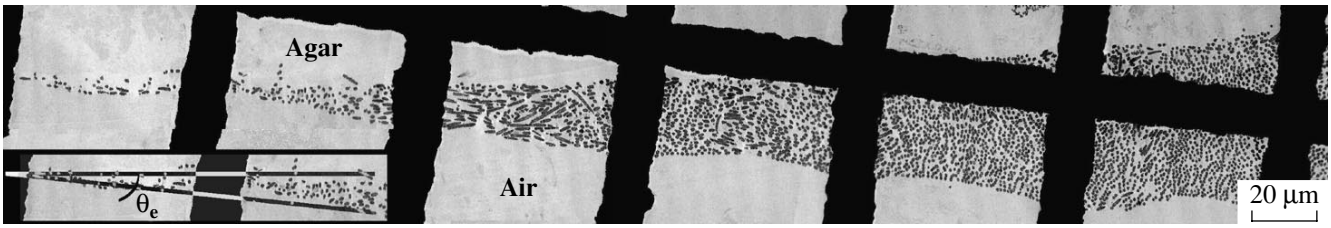

Fig. 2. Scanning transmission electron microscopic (STEM) images of vertical sections on the periphery of the colony grown from a vegetative cell. The images show the distribution of bacterial cells in the colony and its wetting angle $\theta_e = 6°$.

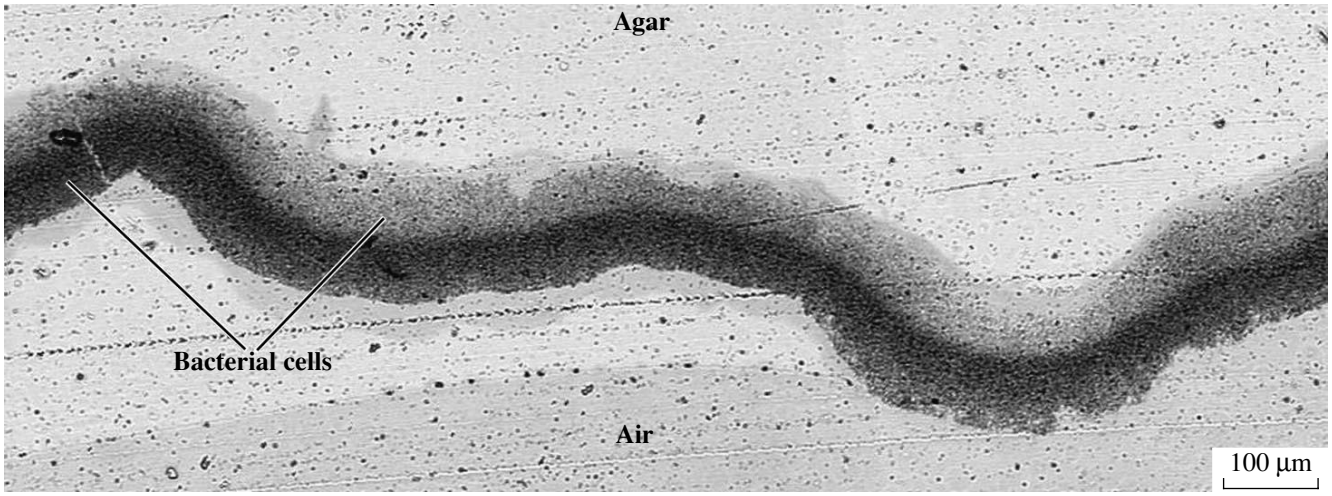

Fig. 3. Light microscopic image of a semithin vertical section of the central part of the colony grown from a germinated spore. Bacterial cells in the colony occur in the form of a layer (or film) located at the colony boundary with the air. The image was obtained with the aid of a Carl Zeiss image processing system.

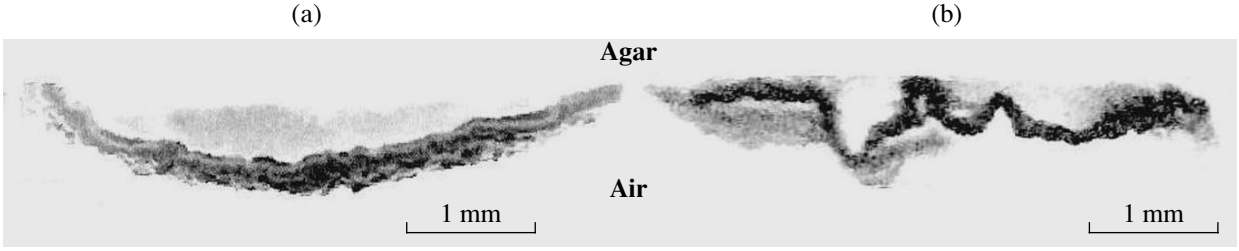

Fig. 4. Profiles of (a) cup-shaped and (b) wrinkled *B. subtilis* 2335 colonies grown from germinated spores. The profiles were obtained with the aid of a DM 23 binocular and a Logitech QuickCam Home video camera.

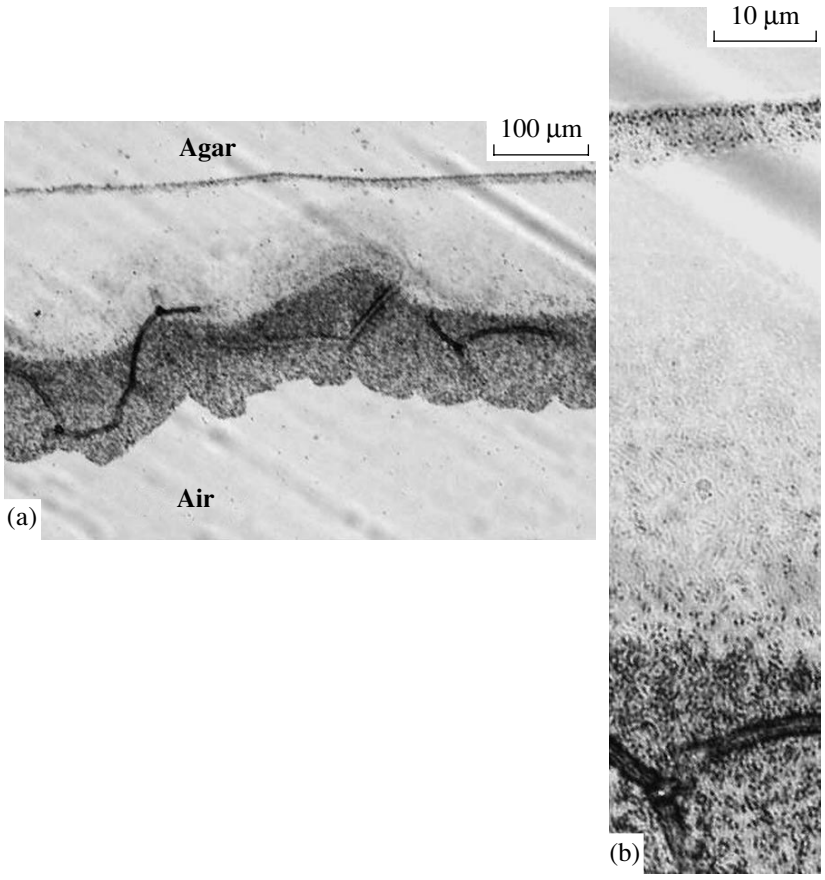

Fig. 5. (a) Light microscopic image of a semithin vertical section of the central part of the colony grown from a germinated spore and (b) a 10-fold magnified fragment of this image. The colony has a layer of bacterial cells adjacent to nutrient agar. The image was obtained with the aid of the Carl Zeiss image processing system.

colonies, one may distinguish ridges and furrows on their surface (Fig. 1b).

RESULTS

The microscopic analysis of the vertical sections of colonies grown from the germinated spores and vegetative cells of *B. subtilis* 2335 revealed two substantial differences in the architectonics of such colonies.

The first difference lies in the fact that bacterial cells in the colony grown from a vegetative cell occupy the entire volume of the colony (Fig. 2), whereas bacterial cells in the colony grown from a germinated spore occur in the form of a layer (or film) located at the colony boundary with the air (i.e., in the bottom part of the colony) (Fig. 3). Such distribution of cells is typical of both cup-shaped and wrinkled colonies (Fig. 4). In a cup-shaped colony, bacterial cells are in contact with nutrient agar only on the periphery of the bottom of the colony (Fig. 4a). In a wrinkled colony, cells are in contact with the agar not only on the periphery of the colony but also where the bottoms of deep furrows are close to the agar surface (Fig. 4b).

The layer of bacterial cells at the boundary with the air is 100–200 μm thick (Figs. 3 and 5). The layer of bacterial cells grown near the bottoms of deep furrows may contain up to 10–15 cell monolayers; however, the internal volume of wrinkled colonies, like that of cup-shaped colonies, does not contain bacterial cells (Fig. 5).

Although the colonies grown from spores and vegetative cells differ in architectonics, the morphology of the cells in these two types of colonies is the same: some cells are structurally intact, some cells are partially degraded, some contain spores, and some are not detached from each other after division and so occur in the form of chains. The colony surface contacting the air is coated by a film (Fig. 6).

The second difference lies in the fact that the wetting angle of the edge of both cup-shaped and wrinkled colonies grown from spores is between 120° and 160° (Fig. 7), whereas that of both types of colonies grown from vegetative cells is between 5° and 30° (Fig. 2).

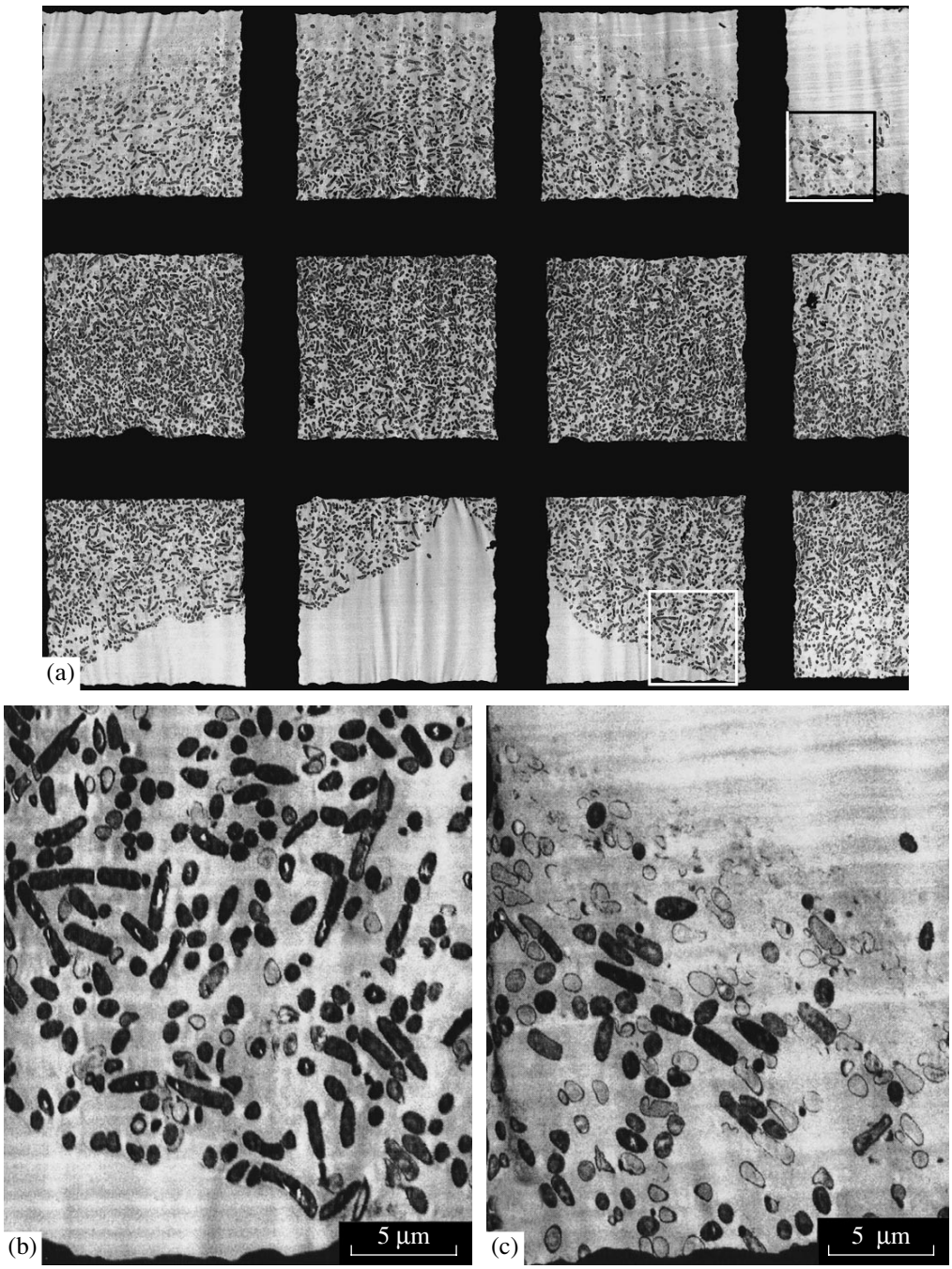

Fig. 6. (a) STEM images of ultrathin vertical sections of the central portion of the colony grown from a germinated spore, (b) a magnified fragment of the colony surface in contact with the air; and (c) a magnified fragment of the colony bulk without bacterial cells.

The wetting angle of colonies can easily be measured on their photographs [6].

The study of the etched vertical sections of colonies revealed a structural difference between nutrient agar and a substance located between it and the layer of bacterial cells in the upper part of the colonies (Fig. 8).

The consistency of the two types of colonies and their adhesion to the nutrient agar were also different. The consistency of the colonies grown from vegetative cells was soft and viscous, whereas the colonies grown from spores were rather solid and poorly adhered to the nutrient agar, so that they could easily be moved over

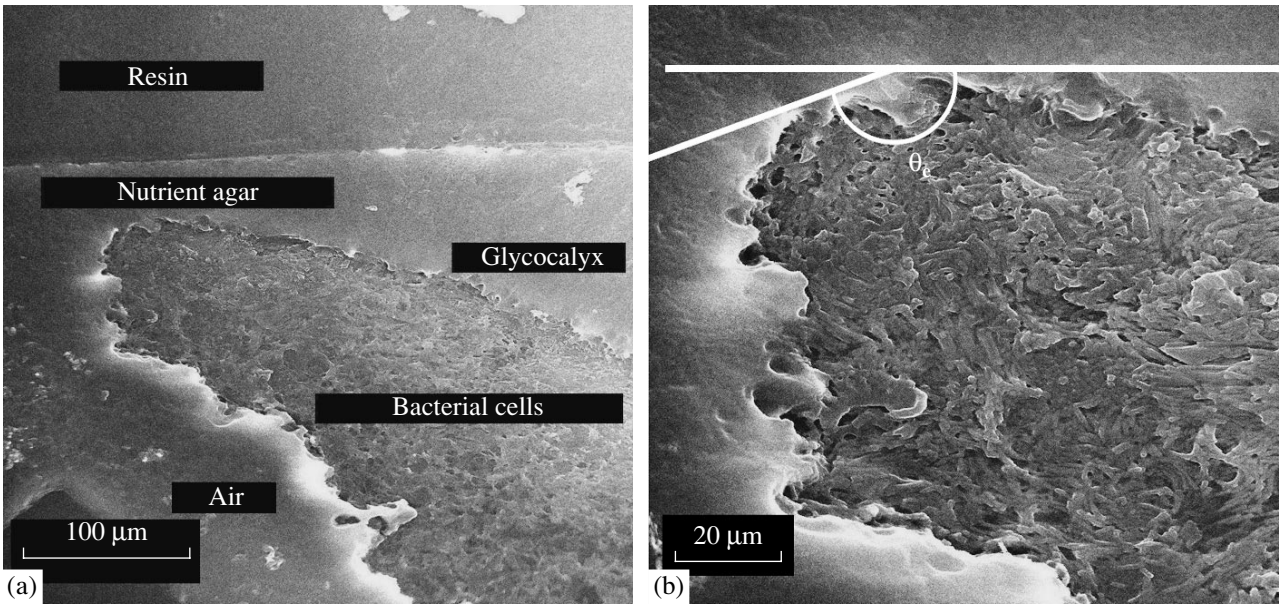

Fig. 7. (a) Scanning electron microscopic image of a vertical section on the periphery of the cup-shaped colony grown from a germinated spore and (b) a magnified fragment of this image showing the wetting angle $\theta_e = 160°$.

the agar surface or detached from it without inflicting damage on the colonies.

DISCUSSION

In the colonies grown from vegetative *B. subtilis* 2335 cells, the latter occupy the whole colony volume (Fig. 2). This is in agreement with the accepted conception of the colony structure. At the same time, the colonies of this bacterium grown from germinated spores had an abnormal structure, which is characterized by the location of cells some distance away from nutrient agar (Figs. 3–5). Actually, such a location of cells is unfavorable for them, since this lengthens the diffusion paths of nutrients present in the agar and metabolites excreted from the cells. To understand the reasons for such cell location in colonies, it is necessary to answer the following questions: What substance separates the nutrient agar and the layer of bacterial cells in the colony? And what hinders bacterial cells from occupying the bulk of the colony? The microscopic methods used by us cannot provide an answer to these questions. However, we can put forward some hypotheses.

The hypothetical substance separating the region of bacterial growth from the nutrient agar (Fig. 8) may represent either (1) a medium saturated with gases evolved by bacterial cells, or (2) nutrient agar partially liquified and degraded by extracellular bacterial enzymes, or (3) bacterial glycocalyx produced by cells present in the colony. Let us discuss these hypotheses.

(1) Hypothesis number one is consistent with the spatial position of growing colonies beneath nutrient agar, as a result of which gases evolved by bacterial cells accumulate in the upper part of the colony, saturating the glycocalyx where dividing bacterial cells occur [7, 8]. The glycocalyx–gas interphase may present an insurmountable barrier for bacterial cells, explaining their absence in the upper part of the colony. However, the saturation of the glycocalyx by metabolic gases must reduce the cross-section of diffusion of nutrients and cell metabolites and thus make the growth conditions of cells in the colony even more unfavorable. Another weak point of this hypothesis is that it suggests the absence of gas diffusion into the nutrient agar and the surrounding air, which seems to be improbable. Therefore, this hypothesis requires substantial experimental underpinning.

(2) The hypothesis that nutrient agar is degraded by extracellular bacterial enzymes also seems unlikely. Indeed, if this is so, colonies would grow inside the agar, which is not the case as evidenced by microscopic observations. Conversely, these observations show that bacterial cells are either absent on the agar surface (Fig. 3) or form a thin layer on it (Fig. 5) but never penetrate into the agar, so that colonies can be moved over the agar surface with an inoculating loop. Furthermore, *B. subtilis* 2335 does not possess agarolytic activity.

(3) The hypothesis that the separating substance is the glycocalyx (the intercellular polysaccharide matrix [7, 9]) of bacterial cells seems the most likely, despite an apparent contradiction lying in the fact that the synthesis of the surplus amount of the glycocalyx requires additional energy and, therefore, must slow down other cellular processes, such as cell growth and division.

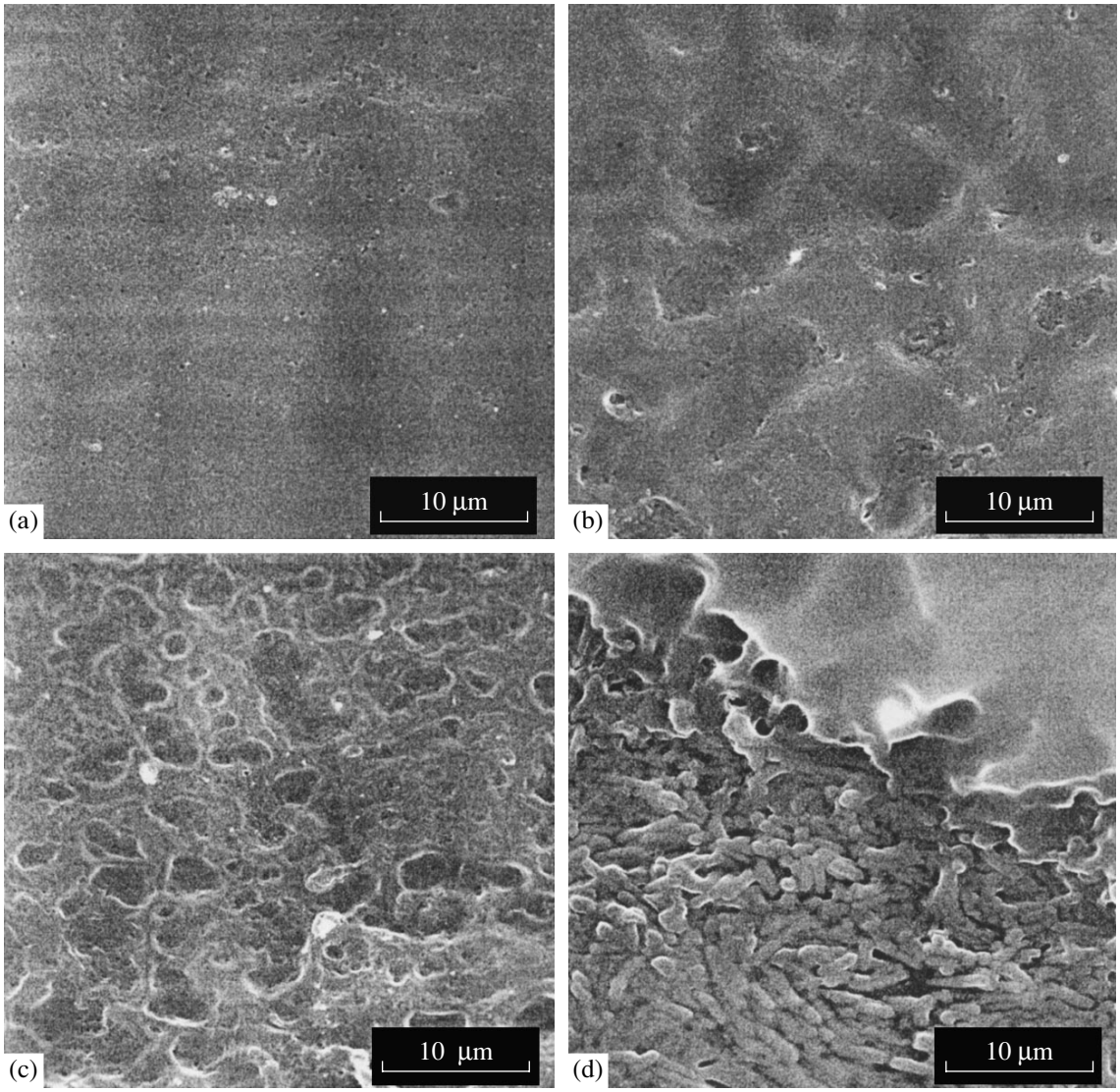

Fig. 8. Changes in the topography of the central part of a colony in a direction from (a) nutrient agar to (d) the colony bottom through (b and c) the colony bulk containing no bacterial cells.

Further consideration of the possible mechanisms responsible for the specific architectonics of the colonies grown from germinated bacterial spores will be performed in terms of the third hypothesis stated above.

We shall consider the following suppositions explaining the presence of bacterial cells in the bottom portion of a hanging colony and their absence in the other portions of the colony:

(1) Taking into account the spatial position of the growing colony, we may suggest that reproducing cells, which possess negative buoyant density and gradually loss their motility, will sink in the glycocalyx, forming a layer at the bottom colony–air boundary.

(2) Another supposition is that initially bacterial cells occupy the entire volume of the growing colony and that by the time of specimen preparation (the fifth to sixth day of cultivation), they undergo lysis. On the one hand, this supposition is in agreement with the observation that bacterial colonies may have an extended zone of cell lysis [10, 11]. On the other hand, our microscopic observations did not detect any traces of cell lysis (such as cell wall remnants) in colonies. Therefore, either this supposition is incorrect, or there exists an unknown type of cell lysis when cells are completely dissolved.

(3) It can be suggested that cells in the colonies grown from germinated bacterial spores are more aerobic than those in the colonies grown from vegetative

cells. This supposition explains the location of cells in the bottom part of the first type of colonies, where the concentration of oxygen is maximum. This supposition is also in agreement with the distribution of lysed cells observed on ultrathin sections (Fig. 6): the percentage of lysed cells gradually increases from the colony–air boundary, where their number is minimal (Fig. 6b), to the colony–agar boundary, where their number is maximal (Fig. 6c). It should be noted that as the amount of lysed cells increases, the intensity of thin section staining usually decreases (Fig. 3).

(4) The uncommon distribution of bacterial cells in colonies can also be explained in terms of wettability. Bacterial glycocalyx is a polymeric material containing up to 99% water [7]. Therefore, the glycocalyx of a colony can be considered as a fluid situated on nutrient agar [12], whose surface must be wetted by the glycocalyx to a degree dependent on the surface tension of the glycocalyx and the wettability coefficient of the agar. When plated on nutrient agar, bacterial cells, because of the high surface tension of their glycocalyx, can initially spread only on the agar surface. But when the energy required to enlarge the surface layer of cells by a unit area becomes greater than the surface tension of the glycocalyx, the cells begin to propagate in a vertical direction.

As can be seen from Figs. 2 and 7, the abilities of the glycocalyx of *B. subtilis* 2335 colonies grown from germinated spores and vegetative cells to wet nutrient agar are very different. As is evident from the wetting angles of the two types of colonies, the glycocalyx of the colonies grown from vegetative cells (wetting angle θ_e = 5°–30°) is hydrophilic and wets the agar surface well. This enables bacillar cells easily spread on the agar surface (Fig. 2). At the same time, the glycocalyx of the colonies grown from germinated spores (wetting angle θ_e = 120°–160°) is hydrophobic and poorly wets the agar surface (Fig. 7). As a result, the propagation of bacillar cells in such colonies is difficult, since their glycocalyxes poorly spread on the agar surface. The colonies grown from germinated spores are rather solid and loosely adhere to nutrient agar, so that they can easily be moved over the agar surface or detached from it without inflicting any damage on them. Inasmuch as cells in such colonies are restricted in their ability to spread on the agar surface, they begin to grow in a direction outward from this surface, while free space between the agar surface and the layer of the growing bacterial cells is filled with the glycocalyx. Presumably, if the layer of growing bacterial cells has no defects, the cells will produce a cup-shaped colony, such as that shown in Fig. 4a. But if this layer contains some defects because of local differences in its thickness and elasticity, the grown colony will be wrinkled (Fig. 4b).

Earlier, we suggested that the shape of colonies depends not only on the type of peripheral growth but also on the surface tension of bacterial glycocalyx [12]. In light of the foregoing discussion, we can suggest that the architectonics of bacterial colonies is also controlled by the degree of the hydrophobicity (or, conversely, hydrophilicity) of the glycocalyx, which, in turn, depends on the type of the inoculum used (in the present work, either spores or vegetative cells of *B. subtilis* 2335).

When vegetative cells are plated onto the surface of nutrient agar, their growth conditions virtually do not change and the cells continue to synthesize glycocalyx with the same properties as before plating to provide for efficient utilization of nutrients. When cells are kept under unfavorable conditions (in the present work, at a low temperature), this induces the production of spores, which presumably bear a genetic program controlling the synthesis of a hydrophobic glycocalyx. In addition to the known properties of glycocalyx, such as those responsible for bacterial virulence and tolerance to heavy metal ions [7], the hydrophobicity of the glycocalyx produced by germinated spores may enhance the survival rate of bacterial cells on the surface of nutrient agar to be colonized. If this supposition is true, the vegetative cells and spores of *B. subtilis* 2335 must have different colonization strategies for the same substrate.

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