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EXPERIMENTAL ARTICLES

Research on the Early Stages of Spore Germination in *Bacillus licheniformis* Using Dynamic Phase Microscopy

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Abstract—The changes in the state of *Bacillus subtilis* spores that occur during germination were analyzed using dynamic phase microscopy (DPM). DPM is based on monitoring and analyzing the interference image of a specimen in a coherent laser beam. The optical path difference (the phase thickness of the specimen, PT) depends on the geometrical height of the specimen and its refractive index. We demonstrated that the maximum PT value is a convenient criterion of the physiological state of the organism involved: PT is ≥ 80 nm, ~40–50 nm, and ≤ 20 in dormant, developing (initiated), and heat-killed spores, respectively. We established that (i) heating a spore suspension to 40°C results in a reversible twofold decrease (from 80 to 40 nm) in their PT under conditions that do not promote the development of the bacteria; this decrease is irreversible under growth-promoting conditions; (ii) the PT values of germinating spores oscillate with a considerable fluctuation amplitude (up to 7 nm), in contrast to the limited fluctuation amplitude (within 1 nm) in dormant spores; (iii) activated spores were heterogenous with respect to the PT pattern: a majority of the spores exhibited a usual spatial profile (with a maximum thickness in the center), whereas a minor fraction of them were characterized by an erythrocyte-like profile with a concave center; this implies that the central zone of the spore was more rapidly hydrated (with a decrease in refractive index) than the peripheral zone.

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Key words: Bacillus licheniformis, spore germination, dynamic phase microscopy, phase thickness, phase thickness fluctuations.

It is widely accepted that most microorganisms occurring in natural habitats exist in a dormant state, as specialized dormant forms (DF) including endo- and exospores, cysts, akinetes [1], cyst-like cells, which have been detected in a wide range of pro- and eukaryotic microorganisms [2, 3], and viable nonculturable cells formed mostly by gram-negative bacteria [4]. DF must retain their viability, i.e., the capacity to revert to active growth and reproduction, although the conditions necessary for this vary depending on the DF type involved. Along with DF, the pool of nondividing and metabolically inactive cells invariably includes both cells that are completely unable to resume their growth but retain their morphological integrity (these have been termed micromummies [5]), and cells that produce one to two generations of offspring under favorable conditions and are undetectable in microbiological tests [6]. Conventional microbiological inoculation methods applied to natural habitats enable us, therefore, to identify only a minority of the total microbial cell number that can be estimated by cell counting. These methods provide only limited information concerning the physiological state of microbial cells in situ. Nevertheless, a prerequisite for efficient ecological, sanitary, and epidemiological research is (i) determining the total number of microorganisms on a natural substrate and (ii) assessing their potential capacity to revert to active metabolism and growth.

This study used endospores of *B. licheniformis*; this organism was chosen because endospores of bacilli are the most extensively studied DF type in microorganisms. Detailed information concerning the physiological, morphogenetic, biochemical, cytological, and genetic aspects of endospore formation and the structural and functional changes associated with their germination was accumulated in the 1960–1990s [6, 7]. Endospore germination includes (i) the reversible activation stage (the spore still possesses properties characteristic of dormancy) that may occur under conditions which do not promote the growth of bacteria; (ii) the irreversible initiation stage characterized by active met-

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abolic processes that are primarily associated with the degradation of spore-specific structures (this stage takes place under growth-promoting conditions and often requires the presence of initiating substances (germinants) in the medium); (iii) the elongation stage that includes second-order stages from growth tube formation to the removal of the spore envelope from the protoplast and to the formation of the first vegetative cell [6]. Research on the germination of these spores and other DF types presents difficulties owing to the high tempo of the initial spore germination stages and to the fact that spores are firm and nevertheless sensitive to mechanical factors. However, this research is of paramount importance, because it helps us find out whether DF are capable or incapable of reverting to the vegetative developmental cycle.

Optical research supplies important information regarding the structural and functional changes that proceed in germinating dormant cells [8]. However, conventional optical microscopy provides limited knowledge. Electron microscopy deals with destroyed objects. The application of fluorescent dyes in fluorescent microscopy is an invasive method that was developed mainly for studying vegetative cells. In studies of dormant cells with disrupted barrier functions of the cytoplasmic membrane, this method can result in artifacts. In addition, conventional optical methods do not enable us to monitor rapid processes. Atomic force microscopy and tunnel microscopy provide high spatial resolution, but studies of living systems that constantly change their state are not always sufficiently efficient [8]. Therefore, developing new methods of investigating rapid metabolic processes in living systems during their transition from the dormant to the active metabolic state is still of particular importance.

In light of these considerations, the method of dynamic phase microscopy (DPM) is of special interest. This method is based on monitoring and analyzing the interference image of a specimen in coherent light, which distinguishes it from the phase microscopy method [8–10]. Measuring wavelength-normalized extremely small (ca. 0.1 nm) changes in the optical path difference allows us to obtain specimen images with a spatial super-resolution [10–12]. The monitored local path difference of the interfering beams $\Delta h(x, y, t)$, referred to hereinafter as phase thickness (PT), reflects the state of the specimen at the t time and varies depending on its position on the plane (on the x and y axes) and in time (the t axis). The method was developed by Prof. V.P. Tychinsky from the Moscow State Institute for Radioengineering, Electronics and Automation, one of the authors of this article. The specimen PT depends on its geometrical thickness and refractive index. The refractive index varies depending on the concentration and properties of the substances contained in the tested specimen and particularly on its hydration degree. Importantly, the terms "geometrical thickness" and "phase thickness" are different. The term "geometrical thickness" denotes the real thickness of the specimen that is on the order of magnitude of micrometers. The "phase thickness" is affected by the optical properties of the specimen and of the immersion medium and is on the order of magnitude of tens of nanometers.

DPM has been successfully used in studies with a number of microscopic biological systems. A relationship between the phase thickness and the metabolic state of the systems involved has been revealed in isolated mitochondria and chloroplasts [13–15].

The goal of this work was to investigate the initial stages of spore germination in *B. licheniformis* using DPM.

MATERIALS AND METHODS

Bacillus licheniformis spores were obtained by growing the bacterium in submerged culture. The medium composition was as follows (g/l): $NH_4H_2PO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; KCl, 0.2; $CaCl_2$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.017; glucose, 0.5%; nutrient broth, 35%; pH 7.0. The culture was grown for 7 days at 28°C on a shaker (180 rpm). Thereupon, the cells were washed to remove the medium and stored for 1–3 months at 20–24°C. The viability of the spores was determined by inoculating tenfold dilutions of their suspensions on nutrient agar and determining the resulting colony-forming unit (CFU) concentrations. Microscopic studies were conducted with a Docuval phase-contrast microscope (Carl Zeiss, Jena, Germany) equipped with a PhilipsToUcamPRO digital camera. Spore size was determined with an ocular micrometer calibrated using a standard ruler. Electron micrographs were prepared with a JEM500 microscope after fixing the spores, embedding them in epoxy resin, and preparing ultrathin sections according to [5].

DPM studies of the spores were conducted with an Airyscan coherent phase-contrast microscope [9, 11, 16]. Specimen images were visualized on the monitor display in the form of the distribution pattern of the local optical path difference (or phase thickness, PT) $h(x, y)$ for each point in a plane arranged perpendicular to the probing light beam. To evaluate the dynamic properties of the tested specimen, we measured the PT by scanning it at regular time intervals (up to 200 times per second for 5–20 min); the scanner moved along a line centered on the spore image (y = const), referred to hereinafter as the "scan line" [9].

Hence, DPM allows us to obtain three-dimensional images of static objects with a high precision and to determine the time course of phase thickness changes along the scan line for the purpose of describing the dynamic changes occurring in the specimen. Since the scan line is chosen arbitrarily, this enables us to focus our measurements on a particular part (compartment) of the tested cell.

The cross section size of a spore, d, and its maximum phase thickness, Δh, (which depends on the dif-

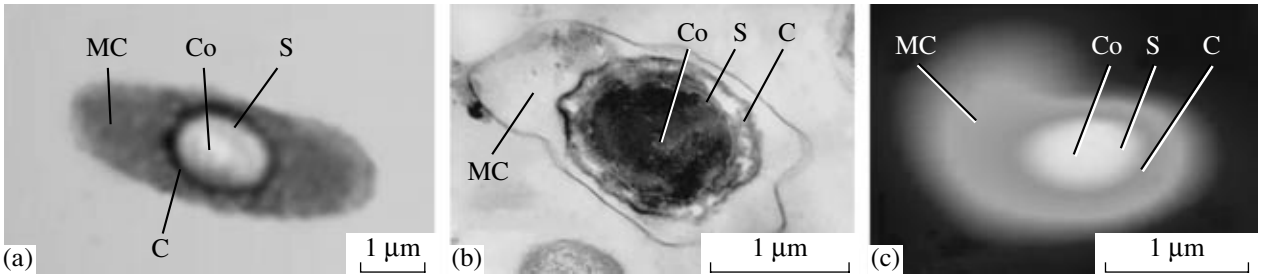

Fig. 1. Images of *B. licheniformis* spores obtained by various methods: phase-contrast microscopy (a), electron microscopy (b), and dynamic phase microscopy (c). Designations: S, spore; MC, mother cell remnants; Co, spore core; C, cortex.

ference between the refractive indices of spores, n, and water (n = 1.33)) [10] were regarded as significant parameters of phase images of spores. The relationship between phase thickness $h(x, y, t)$ and geometrical thickness $H(x, y, t)$ is as follows: $h(x, y, t) \approx \Delta n(x, y, t) \times H(x, y, t)$, where $\Delta n(x, y, t)$ is the difference between the refractive indices (RI) of the specimen and the medium [10]. Our quantitative estimates were based on the assumption that a spore's geometrical thickness H is close to the minimum diameter of an ellipsoid. This enabled us to determine the approximate refractive index averaged for the spore volume. The spore cross-section size value was equal to that determined from phase-contrast images and electron micrographs (~1 μm) (Fig. 1). The maximum RI values (n ~ 1.41) determined by DPM were sufficiently close to the spore RI values reported in the literature [17].

The optical design of the Airyscan microscope used by us [9, 10] is a modified version of the design of the Linnik microinterferometer with a coherent light source (a He–Ne laser, 1 mW, λ = 633 nm) [11]. The linear–periodic modulation of the phase of the reference wave was carried out using a mirror connected to a bimorph. The detection of the interference signal and its AD conversion into local phase values was performed using a coordinate-sensitive photoreceiver (an LI-620 dissector) and an electronic unit. The tested specimen (a spore suspension) was mounted on a polished silicon slide under a cover slip. The measurements were carried out with a 50/0.75 objective. The field size was 4.6×4.6 μm^2. The noise-limited sensitivity h_{min} was 0.1 nm (expressed in optical path difference units). The spectral analysis of PT fluctuations was performed by a standard method fast Fourier transform (FFT).

The temperature of the tested suspension was monitored using a Peltier thermoelement with a thermistor. Spore germination was initiated by adding a fourfold volume of nutrient broth (NB) to the spore suspension prior to the experiment or by adding NB directly to the spore suspension on a silicon slide through a 0.5 mm-wide aperture in the polished silicon slide at the bottom of the cuvette.

Three or more repeats of each experiment were done. The bar chart diagrams contained data obtained with 80–100 spores. The figures contain results that were typical of each experiment. The statistical significance of the results was 0.95.

RESULTS AND DISCUSSION

Comparative study using various microscopic methods. The first series of experiments compared the information supplied by DPM, phase-contrast (PC) microscopy, and electron microscopy (EM). The study was aimed at carrying out a statistical analysis of dormant endospores of *B. licheniformis* (Fig. 1). The computer images obtained by DPM differed from the data obtained using PC microscopy.

The spore images obtained by DPM and PC microscopy (Figs. 1c and 1a, respectively) were bright ellipses surrounded by gray halos corresponding to the remnants of mother cells. The image density in a PC picture is inversely proportional to the optical density of the specimen. Therefore, the two phase images of spores obtained by DPM and PC microscopy are analogous. Both methods yielded similar estimates of the sizes of spores and the remnants of mother cells (1.4–1.6 μm), which conformed to the results obtained by electron microscopy (Fig. 1b). Naturally, the fine details of the internal spore structure visible in an electron microscope could not be revealed using either PC or DPM.

Hence, a comparison of the images given in Fig. 1 indicates that the DPM images are in conformity with the images obtained by conventional methods, because both types of images include the same details and provide the same estimates of the sizes of spores and the remnants of mother cells.

The information supplied by PC microscopy (concerning the sizes and the RI values of various parts of spores) is confined to the above data, whereas DPM enables us to obtain important additional knowledge. This is evident from Fig. 2, which presents three different techniques of optical visualization of the features of spores characterized by different physiological states, including dormant, initiated, and autoclave-killed spores. Spore germination was initiated by adding a fourfold volume of nutrient broth prior to the measure-

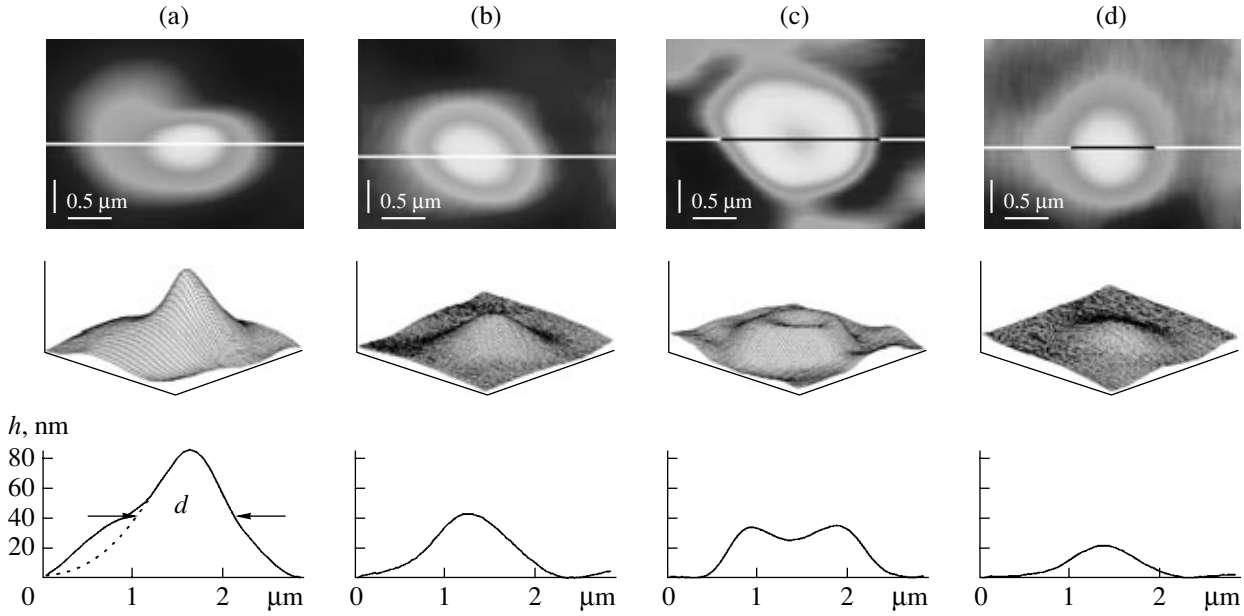

Fig. 2. Images of *B. licheniformis* spores in different physiological states. The images were obtained by dynamic phase microscopy and presented using various techniques. Initiated spores (a), NB-initiated spores with a profile typical of most germinating spores in the population and characterized by a PT maximum in the center (b), NB-initiated spores with a profile occurring in a part of germinating spores stored for three months or less (c), and autoclave-killed spores (d). Upper row, topograms; middle row, three-dimensional images; lower row, phase thickness profiles obtained by sectioning the respective phase images (see text). In the lower-row images, the cross-section size of spores (*d*) is given, determined at the half-height level in the respective PT profile. In the upper-row images, we show the position of the scan line used to determine *d* and *h* (see Materials and Methods). Dotted line, the probable PT profile of the spore upon subtraction of the contribution of the remnants of the mother cell.

ment. The first (upper) row in Fig. 2 contains two-dimensional spore images (topograms) that bear maximum similarity to usual PC pictures. Shades of gray reflect changes in the phase thickness (PT) of the specimens.

DPM enabled us to obtain three-dimensional spore images (middle row, Fig. 2) using quantitative estimates of PT. This conversion fails to yield data on the actual geometrical shape of a cell, but it is informative of the substance distribution pattern within its volume and/or of the dehydration degree of the specimen. The third technique of data presentation is based on plotting the PT profile in the cross-section of the spore image (along the scan line running through its center). We used the PT profile (lower row, Fig. 2) at the half-height (Δh) level to determine the cross-section size (the diameter) of object *d*.

A comparison of the specimens in the middle and the lower row in Fig. 2 reveals that the maximum PT (Δh) drops from 80 to 40 nm during spore germination. In visual studies using PC microscopy, spores darkened during germination, suggesting a decrease in spore refraction. The concordance of the results obtained by two methods (DPM and PC microscopy) was confirmed by analyzing images of autoclave-killed spores (Fig. 2d). The fourfold decrease in the maximum PT of dead spores (from 80 to 20 nm) is related to a marked decrease in their contrast degree, as detected visually by means of PC microscopy (data not shown).

Importantly, the visual differences between the spores in the topograms (Fig. 2, upper row) are not particularly prominent. However, comparison of the numerical PT values (Fig. 2, middle and lower row) reveals substantial differences between spores characterized by different physiological states. The maximum PT of intact, initiated, and dead spores was 80, 40, and 20 nm, respectively. In contrast to PC microscopy, which chiefly deals with qualitative estimates of refraction indices, DPM yields quantitative PT values (available in the digital form) with a very high precision (to within fractions of a percent).

Optical properties of individual spores during germination. DPM enabled us to disclose details of the internal structure of spores that were inaccessible for conventional methods of optical microscopy. It was evident that the whole NB-activated spore population subdivided into two subpopulations several minutes after the activation of its germination. A majority of the spores displayed a usual profile with a maximum PT value in the center and a gradual decrease in PT toward the spore edges (Fig. 2, lower row, b). Some of the spores (15–20%) looked bizarre. They had the shape of ellipsoids with a concave central zone (Fig. 2, lower row, c). Since the actual geometrical shape of a spore is obviously ellipsoid, the data of the figure indicate that the spore structure is optically heterogeneous. Its optical density was low in the center and comparatively high in the cortex zone. We were the first to detect the

Comparison of the parameters of germinating spores of *B. licheniformis* obtained by phase-contrast microscopy and DPM. Spore germination was initiated by adding NB

Tested parameter	Phase contrast	DPM
Direction of changes in optical parameters during germination	RI decreases	PT decreases
Spore size changes	Increases	Increases
Cross-section size of dormant spores, μm	1.6 × 1.4	~1.5
Content of dormant spores, %:		
initially	95–97	95
15 min after activation	50	40
45 min after activation	10	10

Note: The dormant state of spores was detected visually, based on a high refractive index (for PC microscopy) or a high PT value (80 nm and above; for DPM).

optical heterogeneity of a germinating spore. One of the prerequisites for spore germination is spore hydration, which is accompanied by a decrease in optical density. We believe that the ellipsoid shape with a concave center is due to a higher hydration rate and, accordingly, a higher metabolic activity in the spore center compared to the cortex. The hydration rate of the cortex of spores with an erythrocyte-like PT profile was lower than that of the majority of the spores. Spores with the unusual PT profile only occurred in spore preparations whose age did not exceed one month. In suspensions of older spores (with an age of over one month), a quick and homogeneous PT decrease occurred throughout the whole spore profile. The heterogeneity detected by us results from the processes that are accomplished during spore maturation. Their consequences manifest themselves during spore germination.

Hence, PT and spore size values can be used to develop criteria for distinguishing viable, dormant, and dead cells in natural habitats.

Monitoring several spores simultaneously. A significant advantage of DPM is that it enables us to analyze the images of several specimens at the same time. In conjunction with the fast rate of supplying digitized information, this makes it possible to analyze a large number of specimens, which is a prerequisite for obtaining statistically reliable results. This is of paramount importance for investigating rapid biological processes. The Table sums up the data on the properties of a germinating spore population that were obtained by analyzing over 200 spores by means of PC microscopy and ca. 100 spores using DPM.

The data contained in the Table indicate that the results obtained by both methods are identical. They suggest that DPM is an effective method for use in studies with unstained biological systems (spores and cells).

DPM provides valuable information concerning the population-level parameters related to spore germination, including the maximum PT value and the spore diameter in the following systems: dormant spores–NB-initiated spores–autoclave-killed spores. The statistical treatment of the properties of ca. 100 spores revealed the heterogeneity of their population in terms of both criteria (maximum PT value and spore cross-section size). The dormant spore population was dominated (85%) by spores with high PT values (70–90 nm); the spores were highly homogeneous in terms of their size. The initiated spore population was more homogeneous in PT; PT equaled the average value (35–40 nm) in 95% of the initiated spores. However, the heterogeneity of the initiated cell population in terms of their size increased owing to the formation of large cells. The autoclave-killed spore population was strikingly different: it displayed low PT values (20–30 nm) and comparatively small spore sizes.

Importantly, such data on the spore distribution within a population, characterized by rapid processes typical of spore germination, cannot be obtained using PC or fluorescent microscopy or by other conventional light microscopy methods. Nevertheless, such data are of paramount importance both for monitoring spore populations and for evaluation of the quality of the spore inoculum in biotechnological processes. The heterogeneity of spore suspensions was confirmed by electron microscopic studies. Apart from intact dormant spores, these studies revealed defective, germinated, and nonstandard spores (data not shown). However, this method was considerably less advantageous than DPM in terms of speed, effort, and convenience.

Dynamics of heat-induced changes in the optical properties of spores. Since a fraction of the spore population displayed a PT value below 60 nm, the question to raise was whether these spores were reactivated or dead. The prerequisite for choosing between these alternatives is to obtain additional information by, e.g., monitoring the dynamics of the changes (if they occur) in spore parameters over time.

The following section of this work presents data related to the dynamics of the initial stages of spore germination obtained by DPM.

Spore germination is known to include a reversible activation stage that is difficult to detect. In our experiments, spores were activated by changing the temperature. We used a spore suspension incubated in a medium that was unfavorable for bacterial growth. The spore suspension was gradually heated from 18 to 75°C at a rate of 1–2°C/min directly in a measuring cuvette that was mounted on the microscope stage. Monitoring the behavior of a single spore revealed that the changes in its maximum PT caused by a temperature increase were not monotonous. Fig. 3 demonstrates that extreme PT values were attained at three temperature points (35,

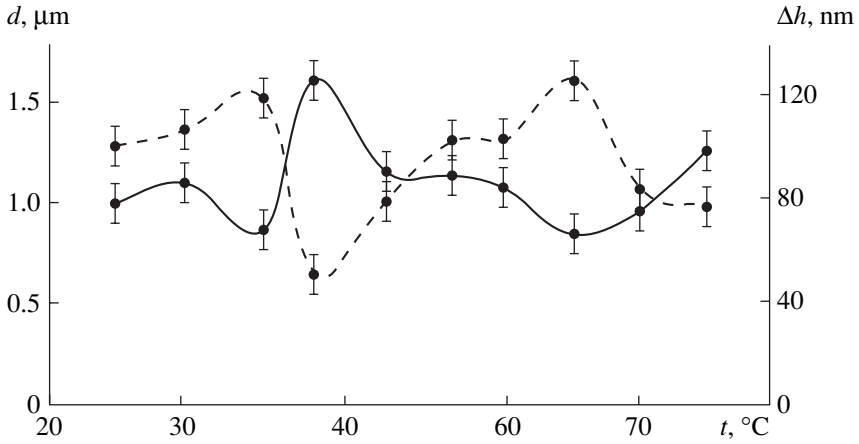

Fig. 3. Temperature dependence between the diameter (d, Δh, dotted line) of a single *B. licheniformis* spore.

38, and 65°C). The high initial PT value (100 nm) remained constant within the 25–35°C range and drastically decreased to 45 nm upon heating to 38–40°C. Upon a further temperature increase to 65°C, the spore PT monotonously increased to the initial value. The second PT maximum occurred at 65°C, and further heating resulted in a PT decrease. The changes in cross-section size (d) in response to temperature changes were opposite to those in PT.

Interestingly, this dynamic of the Δh and d values was characteristic of an overwhelming majority of the tested spores. In a small number of spores, the PT decrease at 40–42°C was irreversible, i.e., they started their developmental cycle.

While the PT decrease within the 65–75°C range can be considered a result of spore activation, the Δh decrease at 38–40°C is a new phenomenon that was not observed earlier. The drastic PT change reflecting an alteration in the spores' state upon a very insignificant temperature change (3–4°C) indicates a cooperative pattern of the processes resulting in the PT change.

Since the events observed by us are reversible, they may be associated with spore activation processes. However, it is characteristic of native spores that they retain the properties of the dormant state including, in the first place, cell dehydration. Taking into account the fact that the substance quantity remains unchanged after spore activation, we can explain the significant, yet reversible, PT changes in the spores by postulating a cooperative phase transition of the membranes (lipids) and, possibly, also by conformational changes in integral membrane proteins. In order to elucidate the effects revealed by us, further studies must be conducted in model systems. This will allow us to discern the contributions of the (de)hydration degree of the specimen, the fluidity/crystallinity of membrane lipids, and the conformational changes in macromolecules to the resulting PT changes.

Based on a general analysis of the data on spore parameter changes during heating under conditions that do not favor spore development, we can draw the conclusion that the temperature of 42°C is a prerequisite for retaining the dormant state. This fact was not known before our study. It was only known that thermal spore activation occurs at 65–75°C [6]. It also should be noted that the above-mentioned activating temperature values are characteristic of the spores of *B. licheniformis* used in this work. They may differ to some extent from those that activate the spores of other microbial species. This issue shall be addressed in further studies.

Phase thickness oscillations. Another indisputable advantage of the DPM method is that it enables us to scan a specimen optically with a high frequency (up to 200 Hz) and to monitor dynamic processes either in a single spore or in several spores simultaneously [10]. In our studies, we recorded and analyzed PT changes in single spores that were either in a dormant state or initiated by the addition of a complete nutrient medium (NB). Fig. 4 presents the results of measuring the Δh of a single spore for 30 s; the intervals between measurements were 10 ms. The amplitude of PT fluctuations in dormant spores (Fig. 4, *1*) was close to that of the instrument noise (they did not exceed 1 nm). Fifteen minutes after the activation of spore germination, we observed an increase in the fluctuation intensity that reached 7 nm (Fig. 4, *2*), in addition to a characteristic Δh decrease (from 82 to 52 nm). The Fourier spectra of the fluctuations, whose maximum frequency reached 5 Hz, contained spectral components with frequencies of 1.3 and 3.4 Hz (Fig. 4, insert). The presence of components with frequencies below 0.5 Hz is due to factors unrelated to the tested specimen. Minimum PT fluctuations occurred in autoclave-killed spores, and their spectra lacked contrasting components (data not shown). At present, we cannot provide a biophysical explanation for the PT fluctuations. However, the fact that they occur in developing spores is of particular importance. It provides an additional feature (descrip-

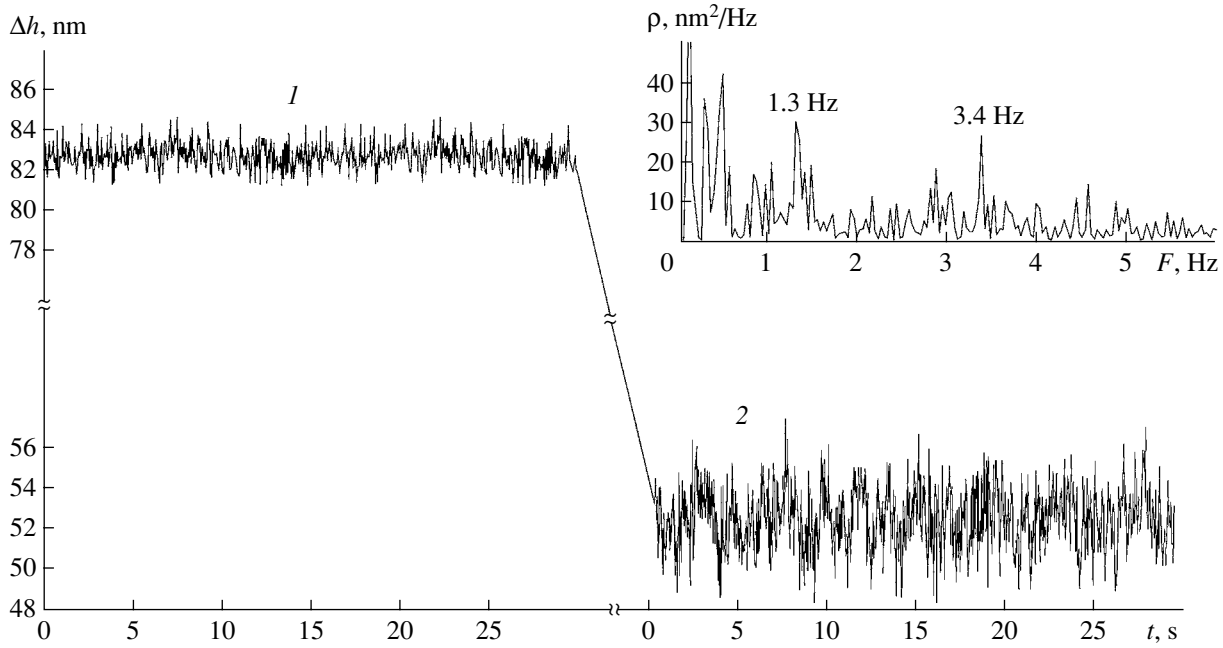

Fig. 4. Time course of phase thickness changes in a dormant (*1*) and nutrient medium-initiated (*2*) *B. licheniformis* spore. Phase thickness was measured for 30 s, as shown on the *x* axis. Insert, the Fourier spectrum of PT fluctuations in an initiated spore; the contrasting components (1.3 and 3.4 Hz) are indicated.

tor) distinguishing an ametabolic spore from a developing spore. Importantly, the PT oscillations observed by us are interrelated in terms of space and time. This is indicative of a cooperative pattern of the processes involved and is possibly due to synchronous changes in the conformational states of macromolecules in a spore. This fact points to a relationship between the processes which share a common causal factor that occur in various scan line points. For example, they may be caused by the operation of enzyme complexes of the respiratory chain or of ion channels and by changes in proton concentration. This whole issue shall be addressed in a special study. Interestingly, analogous fluctuations occur in energized mitochondria [13], cyanobacteria, cells of mammals (*Vero* and *Mammae*), and chloroplasts [14]. The PT fluctuations in initiated spores revealed by us should be regarded as a valid criterion of metabolism (in addition to a PT decrease).

The criterion of spore viability (PT fluctuations) and the distinction between viable and non-viable cells (dynamics of PT changes under growth-promoting conditions) revealed in this work can be used to develop methods for the detection of living organisms in natural habitats including the systems investigated in astrobiological studies.

The analysis of the data presented by us demonstrates that DPM is a highly efficient and informative method applicable to studies on the functional state of native biological systems. Interference images obtained by probing a specimen with coherent laser light with a specific wavelength provide for a high reproducibility of the data. Importantly, these data do not vary depending on the intensity of the light source or the sensitivity and stability of the parameters of the photoreceiver setup.

Using the PT value instead of the refractive index (RI) employed in phase-contrast microscopy provides new insights into the state of tested systems, including spores, enabling us to use numerical (not qualitative) criteria. The spore RI changes from 1.41 to 1.35, i.e., decreases by 5%, during germination, while the PT value varies within the (80–90)–(20–30) nm range, i.e., changes by a factor of 3–4. In terms of the above criterion, this enhances the precision of detection (discrimination) of spores in different physiological states, including dormant, metabolically active, and nonviable spores. An additional advantage of this method is the convenience and high rate of PT determination in individual systems and their groups (in contrast to PC microscopy).

Monitoring PT changes not only within the specimen-scanning plane but also along the time axis, in conjunction with a high scanning rate (up to 200 measurements per second), gives us a unique opportunity to carry out in vivo research on the dynamics of fast processes, including those involving cooperative transitions between phase states of intracellular structures and biopolymers.

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