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EXPERIMENTAL ARTICLES

Fluorescence Microscopic Study of Microorganisms Treated with Chaotropic Agents

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Abstract—The yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* and the bacteria *Micrococcus luteus, Bacillus subtilis*, and *Anaerobacter polyendosporus* have been treated with the chaotropic agents guanidine hydrochloride and guanidine thiocyanate and certain detergents and studied using fluorescence microscopy. Studies with the use of fluorochromes that can selectively stain nucleic acids (diamidino-2-phenylindole (DAPI), propidium iodide, and acridine orange) show that treatment of the bacterial and yeast cells at 37°C for 3–5 h induces a release of DNA from the cytoplasm and its accumulation in the cellular zone, known as ectoplasm, located between the cell wall and the remainder of the cytoplasm (called endoplasm) in the form of one or several large granules. After treating the cells with the chaotropic agents at 100°C for 5–6 min, the DNA is diffusively distributed over the ectoplasm. The fluorochromes used do not allow the detection of RNA. These findings are in agreement with previous data obtained from electron microscopic study of thin cell sections. After 33 PCR cycles, a considerable portion of DNA leaves the cells; as a result, they show a low level of diffusive fluorescence when stained with DAPI. When endospores of *B. subtilis* are treated with the chaotropic agents, they become highly permeable to the fluorochromes. Fluorescence microscopic study of such endospores shows that they contain DNA in the central part of their cores.

Key words: fluorescence and electron microscopy of cells, localization of chromatin substance, treatment of microorganisms with chaotropic agents.

Our earlier electron microscopic studies showed that the treatment of certain yeast and gram-positive bacterial cells with the chaotropic agents guanidine hydrochloride (6 M) and guanidine thiocyanate (4 M) at 37°C for 3–5 h or at 100°C for 5–6 min substantially affects the cell structure, causing a release of chromosomal DNA from the cytoplasm and its accumulation in the ectoplasm, a newly formed cellular zone [1]. The release of DNA is due to degradation of the membrane system. The modified cytoplasm appears as a central electron-opaque zone in which ribosomes and membrane structures are not detectable. The cell wall retains its integrity, and, as a result, high-polymeric DNA remains in the cells [2].

This paper describes the results of fluorescence microscopic study of the effect of chaotropic agents on microbial cells under the same conditions that have previously been used in electron microscopic studies [1].

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MATERIALS AND METHODS

Microorganisms and cultivation conditions. The experiments were carried out with two yeasts (*Saccharomyces cerevisiae* Y-190 IBKh and *Pichia pastoris* GS115 IBKh) and three gram-positive bacteria (*Micrococcus luteus* NCIMB 13267, *Bacillus subtilis* VKM B-504, and *Anaerobacter polyendosporus* VKM B-1726).

The yeasts were cultivated at 30°C for 3 days on YPD agar containing (%) yeast extract, 1; bactopeptone, 2; glucose, 2; and agar, 1.5 (pH 5.3). The anaerobic bacteria were cultivated on nutrient agar at 29°C for 3 days. *B. subtilis* spores were harvested after 10 days of cultivation. *A. polyendosporus* was grown on potato agar following the method described in [3]. Vegetative cells and spores of this bacterium were harvested after 5 and 10 days of cultivation, respectively.

Treatment of the cells with chaotropic agents was performed according to the method described in [1].

Epifluorescence and phase contrast microscopic studies were carried out using LUMAM-2, MBI-15U

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(both from LOMO, Russia) and Polyvar (Reichert) microscopes.

The cells were stained with three fluorochromes: diamidino-2-phenylindole (DAPI) and propidium iodide 9 (Molecular Probe, Inc.), and acridine orange (manufactured in Russia). The procedures were as follows:

(1) A solution of diamidino-2-phenylindole (2 μg/ml) in a phosphate buffer (pH 7.0) was mixed with an equal volume of cell suspension, and the mixture was incubated for 5 min. The specimens were examined under the microscopes using a 365-nm excitation filter.

(2) A solution of propidium iodide (60 μM) in distilled water was mixed with an equal volume of cell suspension, and the mixture was incubated for 10 min. The specimens were examined under the microscopes using a 490-nm excitation filter.

(3) A solution of acridine orange (0.1 mM) in a phosphate buffer (pH 4.5) was mixed with an equal volume of cell suspension, and the mixture was incubated for 10 min. The specimens were examined under the microscopes using a blue excitation filter.

Control cells were treated at 37°C for 1 h with a solution containing 1 mg/ml of micrococcal nuclease (Millipore).

Microscopic images were recorded using Mikrat-200, RF-3, and Kodak 400 films, as well as a CoolPix 995 (Nicon) digital camera connected to a Pentium 5 PC (128 Mb) equipped with a Sapphire Radeon 9800 Pro videocard and an Epson Stylus C82 printer.

Electron microscopic studies were carried out as described in [1].

PCR amplifications were run with yeast-specific universal 18S rRNA primers [2].

RESULTS

Study of the localization of nucleic acids in microbial cells treated with chaotropic agents and then stained with DAPI (selectively binds to DNA), as well as propidium iodide and acridine orange (which bind to both DNA and RNA), provided the following data:

(1) In the *S. cerevisiae* and *P. pastoris* cells treated with the chaotropic agents at 37°C and then stained with DAPI, DNA is localized on the cell periphery in the form of granules (Figs. 1a–1c, 2a, 2b). Most of the *S. cerevisiae* cells contain one large fluorescent polar granule, whereas the *P. pastoris* cells mostly contain 2–4 granules. A comparison of the images obtained by fluorescence and phase contrast microscopic methods showed that the DNA granules are localized between the cell wall and the centrally located modified cytoplasm. This is particularly clearly seen on photographs obtained from a combination of fluorescence and phase contrast microscopic images (Fig. 1c). These data agree well with earlier electron microscopic observations [1]

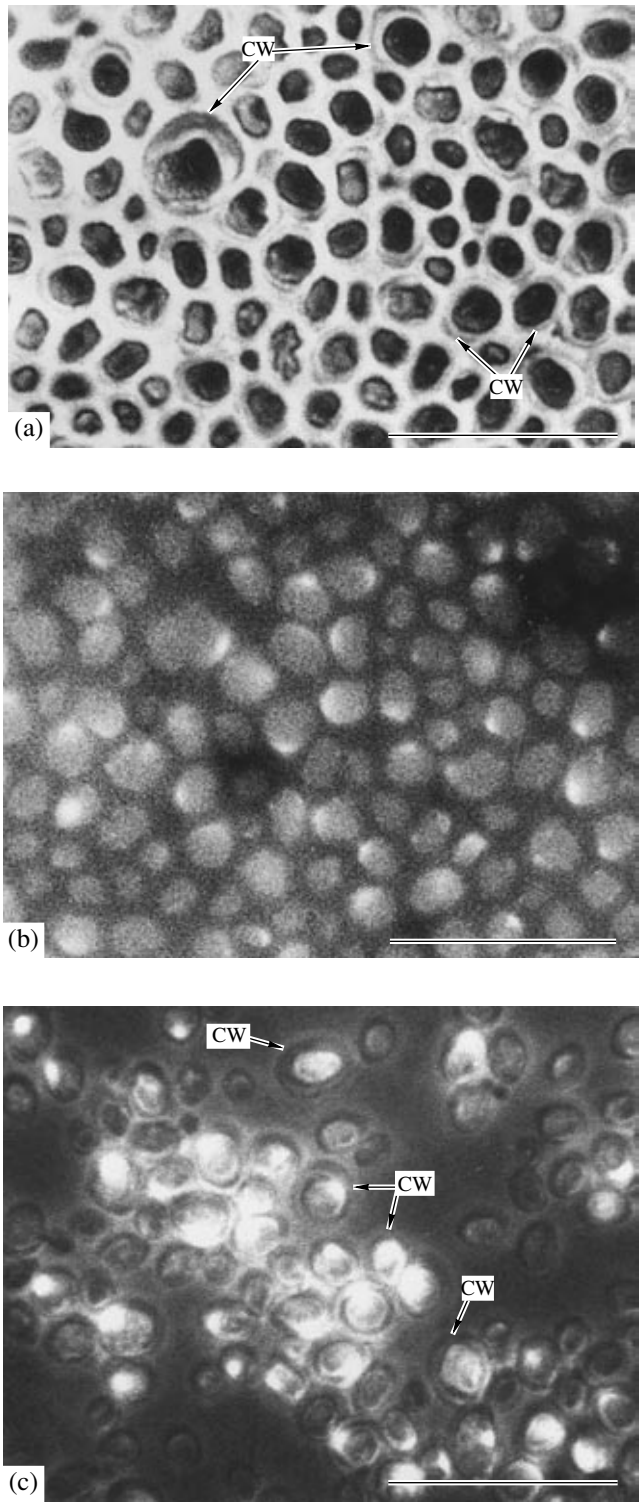

Fig. 1. Images of *S. cerevisiae* cells treated with the chaotropic agents in buffer D [1] at 37°C for 5 h. The images were obtained by (a) phase contrast microscopy, (b) epifluorescence microscopy of DAPI-stained cells (the light regions represent the chromatin substance), and (c) a combined phase contrast and epifluorescence technique (the cell walls and the light regions of the chromatin substance are visible). CW stands for "cell wall." The scale bars represent 10 μm.

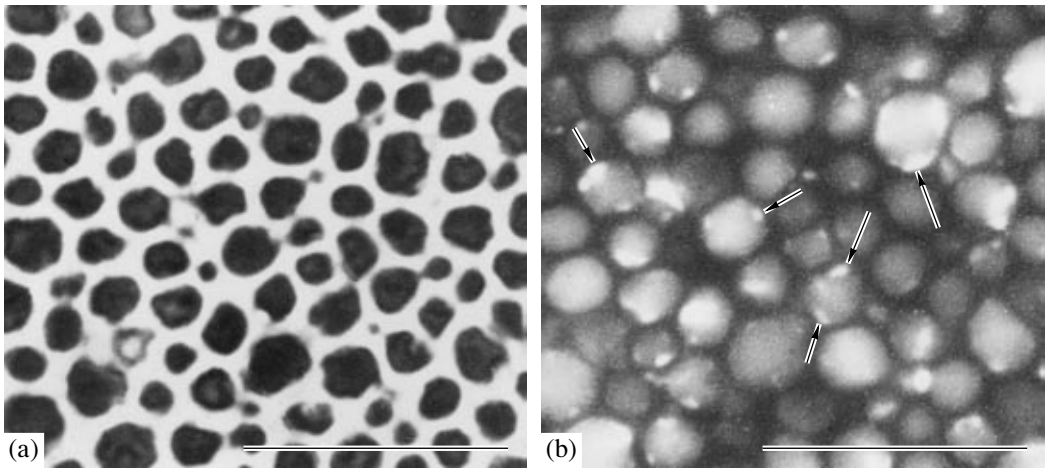

Fig. 2. Images of *P. pastoris* cells treated with the chaotropic agents in buffer D [1] at 37°C for 5 h. The images were obtained by (a) phase contrast microscopy and (b) epifluorescence microscopy of DAPI-stained cells (the arrows point to the chromatin aggregates). The scale bars represent 10 μm.

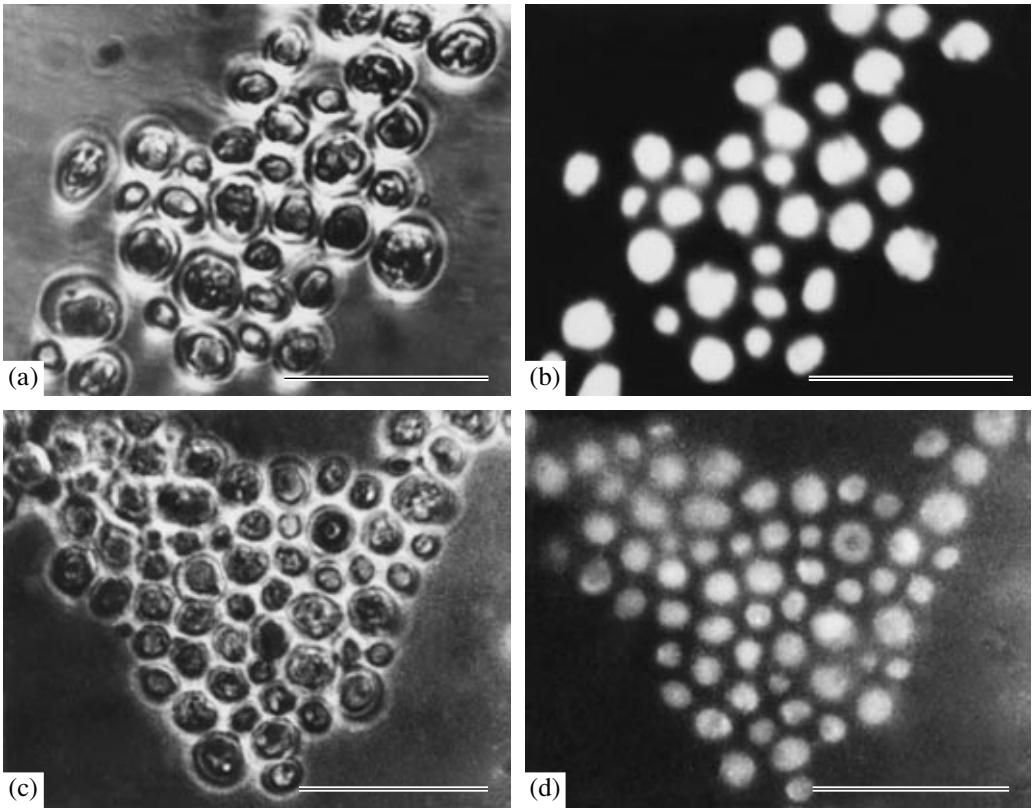

Fig. 3. Images of (a, b) *S. cerevisiae* and (c, d) *P. pastoris* cells treated with the chaotropic agents in buffer D [1] at 100°C for 5 min. The images were obtained by (a, c) phase contrast microscopy and (b, d) epifluorescence microscopy of DAPI-stained cells (the fluorescence of the whole cytoplasm is seen). The scale bars represent 10 μm.

showing that fibrillar material that is contrasted with uranyl acetate and can be removed by nucleases is localized in the ectoplasm, a newly formed cellular zone between the cell wall and the rest of the cytoplasm (called endoplasm). The low uniform fluorescence of cells may be due to the diffuse distribution of a portion of DNA over the ectoplasm.

Fluorescence microscopy of the cells treated with propidium iodide provided similar images. With this

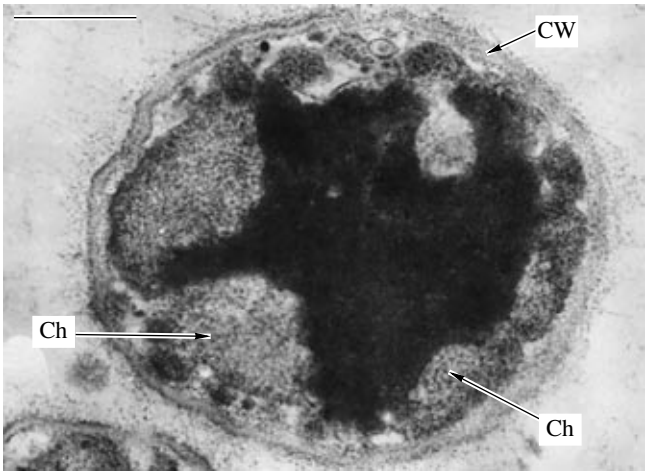

Fig. 4. Electron microscopic image of a thin section of a *P. pastoris* cell treated with the chaotropic agents in buffer D [1] at 100°C for 5 min. CW and Ch mark, respectively, the cell wall and chromatin aggregates in the ectoplasm. The scale bar represents 0.5 μm.

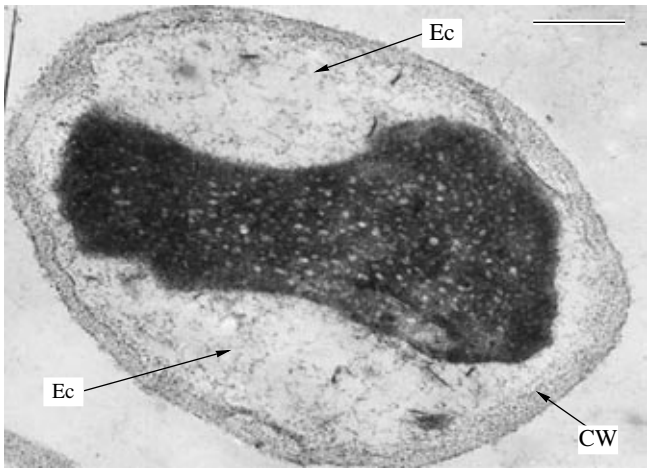

Fig. 5. Electron microscopic image of a thin section of an *S. cerevisiae* cell treated with the chaotropic agents in buffer D [1] at 100°C for 5 min and then subjected to 33 PCR cycles. The electron-transparent ectoplasm contains a net of thin fibrilla. CW and Ec mark, respectively, the cell wall and the ectoplasm. The scale bar represents 0.5 μm.

fluorochrome, however, red diffuse fluorescence of the endoplasm was not observed.

UV and blue-light illumination of the cells gave rise to a yellow–green fluorescence of the cytoplasm, which was probably due to an autofluorescence of the cells or the fluorescence of the chaotropic agents (specifically guanidine thiocyanate) firmly bound to the cytoplasm.

(2) The fluorescence of the yeast cells treated with the chaotropic agents at 100°C and then stained with DAPI was characterized by a homogeneous fluorescence of the whole cell interior (Figs. 2a, 2b, 3a–3d). According to the electron microscopic studies reported in [1], when the chromatin material in the yeast cells is treated with the chaotropic agents at 37°C, it has a high electron density and the chromatin DNA appears as spirally arranged fibrillar bundles (Figs. 6, 7c in [1]). In contrast, according to our fluorescence microscopic studies of the cells after being treated with the chaotropic agents at 100°C, DNA appears as tangles made of a loose net of thin fibrilla (Fig. 4). After 33 PCR cycles, the ectoplasm of the *S. cerevisiae* cells had a net of rare fibrilla (Fig. 5). The intensity of the DAPI-induced fluorescence of the cells was considerably lower (Fig. 6) when they were not subjected to PCR procedures (Figs. 3a–3d).

(3) The *S. cerevisiae* cells treated with the chaotropic agents, *Micrococcus* nuclease, and then DAPI showed no fluorescence.

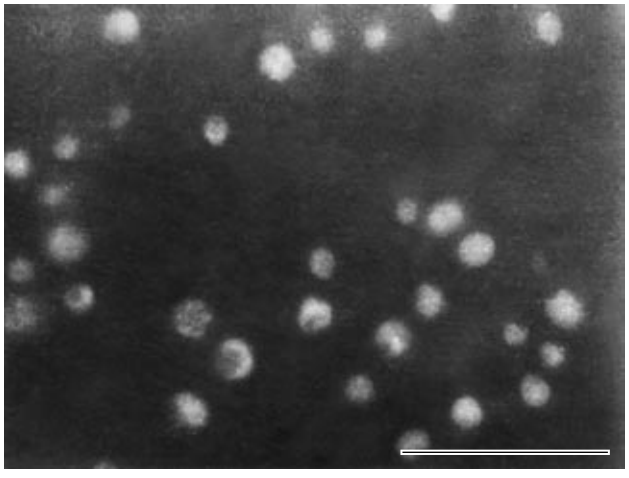

Fig. 6. Epifluorescence microscopic image of *S. cerevisiae* cells treated with the chaotropic agents in buffer D [1] at 100°C for 5 min and then subjected to 33 PCR cycles. The cells were stained with DAPI. The scale bar represents 10 μm.

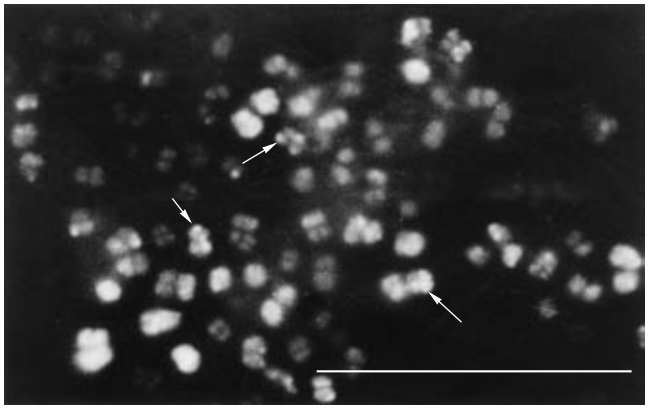

Fig. 7. Epifluorescence microscopic image of *M. luteus* cells treated with the chaotropic agents in buffer D [1] at 100°C for 5 min and then stained with DAPI. The arrows point to light granules of the chromatin substance. The scale bar represents 10 μm.

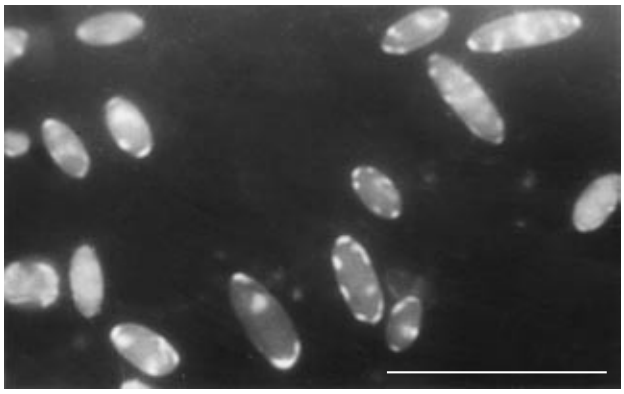

Fig. 8. Epifluorescence microscopic image of *A. polyendosporus* cells treated with the chaotropic agents in buffer D [1] at 100°C for 5 min and then stained with DAPI. The light laterally arranged granules of the chromatin substance are seen. The scale bar represents 10 μm.

(4) The treatment of the *M. luteus, B. subtilis*, and *A. polyendosporus* cells with the chaotropic agents also resulted in redistribution of the nuclear material. According to both electron and fluorescence microscopic observations, this material is localized on the cell periphery between the cell wall and the rest of the cytoplasm (Figs. 7, 8).

(5) Treatment of the *B. subtilis* and *A. polyendosporus* endospores with the chaotropic agents at 100°C for 7 min made them nonrefractive, with the endospores looking like dark germinating spores in the positive phase contrast microscope (Figs. 9a, 9b). Moreover, treatment of these endospores with the chaotropic agents [1] make their partially degraded envelopes and membrane structures permeable to stains, and, as a result, the DNA can easily be seen in the spore cores (Fig. 9c). It should be noted that the electron microscopic studies reported in [1] showed that the treatment of spores with the chaotropic agents does not affect their cortex and nucleoid-containing core.

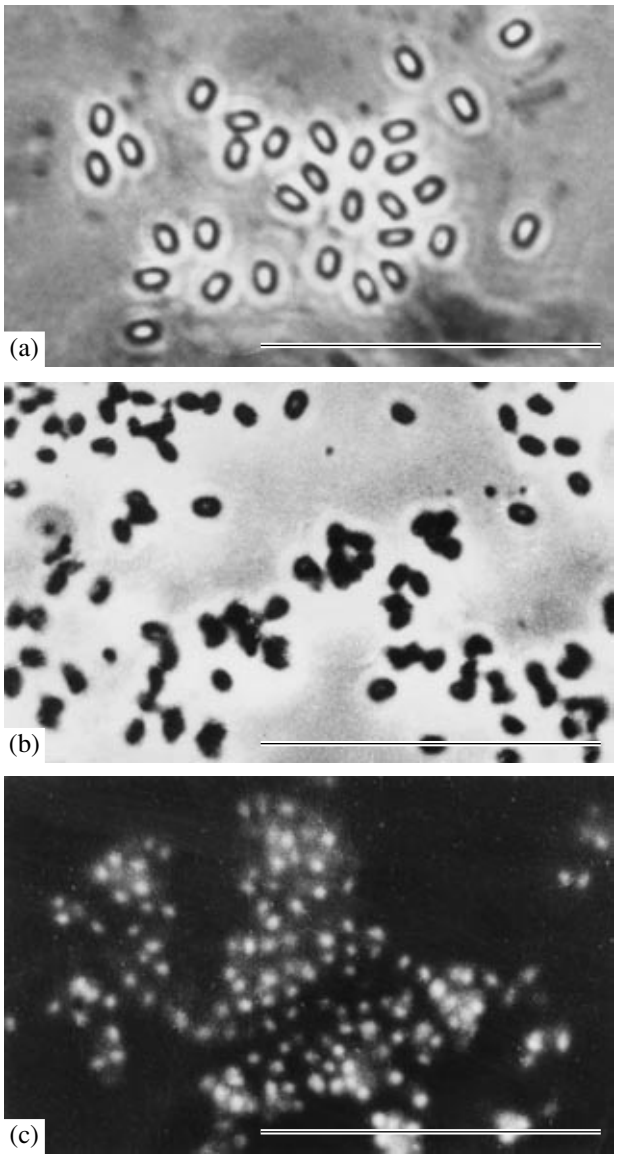

Fig. 9. Microscopic images of *B. subtilis* spores: (a) phase contrast of intact mature spores, (b) phase contrast of spores treated with the chaotropic agents in buffer D-2 [1] at 100°C for 7 min, and (c) epifluorescence microscopy of spores treated with the chaotropic agents in buffer D-2 [1] and then stained with DAPI. Light nucleoids in the spore centers are seen. The scale bar represents 10 μm.

DISCUSSION

The chaotropic agents guanidine hydrochloride and guanidine thiocyanate are suitable for the permeabilization of microbial cells that possess thick cell walls and contain chromosomal DNA. These chaotropic agents are able to fix the cells and remove a considerable fraction of cellular lipids and proteins at a time [2]. The chaotropic agents can rapidly denature cellular protein, including nucleases (see the relevant references in [2]). Cell specimens treated with the chaotropic agents according to the Danilevich and Grishin method [2] are suitable for further biochemical studies and PCR analysis. Electron microscopic examination of microbial cells treated with the chaotropic agents showed that such treatment greatly degrades the cell membranes but leaves intact the cell wall, part of the modified cytoplasm, and the DNA-containing nucleoplasm [1].

The fluorescence microscopic studies described in this paper confirm previous electron microscopic observations showing that when microbial cells are treated with these chaotropic agents, they can retain their DNA [1]. The fact that the chaotropic agents can permeabilize mature bacterial spores to the fluorescent dyes DAPI, propidium iodide, and acridine orange, leaving DNA inside the spores, suggests that these cells can be used in PCR studies.

ACKNOWLEDGMENTS

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