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EXPERIMENTAL ARTICLES

Changes in the Fine Structure of Microbial Cells Induced by Chaotropic Salts

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Abstract—The electron microscopic examination of thin sections of cells of the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* and the gram-positive bacteria *Micrococcus luteus* and *Bacillus subtilis* showed that cell treatment with the chaotropic salts guanidine hydrochloride (6 M) and guanidine thiocyanate (4 M) at 37°C for 3–5 h or at 100°C for 5–6 min induced degradative processes, which affected almost all cellular structures. The cell wall, however, retained its ultrastructure, integrity, and rigidity, due to which the morphology of cells treated with the chaotropic salts did not change. High-molecular-weight DNA was localized in a new cell compartment, the ectoplasm (a peripheral hydrophilic zone). The chaotropic salts destroyed the outer and inner membranes and partially degraded the outer and inner protein coats of *Bacillus subtilis* spores, leaving their cortex (the murein layer) unchanged. The spore core became accessible to stains and showed the presence of regions with high and low electron densities. The conditions of cell treatment with the chaotropic salts were chosen to provide for efficient in situ PCR analysis of the 16S and 18S rRNA genes with the use of oligonucleotide primers.

Key words: ultrastructure of microorganisms, chaotropic salts, membranes, envelopes, cell walls, chromatin, PCR in situ.

It is known that chaotropic salts, such as guanidine hydrochloride and guanidine thiocyanate, exert specific effects on live microbial cells, causing the degradation of membranes, ribosomes, and other cell structures and inducing a release of DNA from the cells [1–5]. For this reason, some chaotropic salts are used to isolate DNA and RNA from gram-negative bacteria and animal cells and tissues [1–4]. There is evidence that chaotropic salts rapidly denature cellular proteins, including nucleases.

Recent studies showed that microbial cells with thick and tough cell walls (yeasts, mycelial fungi, and gram-positive bacteria) are not completely degraded and retain their shape even when boiled in chaotropic salt solutions [5]. The incubation of such cells in the presence of chaotropic salts at 20–100°C extracts most cellular proteins, lipids, RNA, and low-molecular-weight compounds. At the same time, high-molecular-weight genomic DNA, which can serve as a template in PCR reactions [5] and is accessible to nonspecific micrococcal nucleases and specific restriction endonucleases, is retained in cells that are treated with chaotropic salts [8].

The fine structure of chaotropic salt–treated cells (namely, the cellular location of nucleic acids and the structural state of the cell wall, membranes, and other cellular structures) is as yet poorly studied. This prompted us to investigate the structural organization of vegetative and resting cells of eukaryotic and prokaryotic microorganisms treated with chaotropic salts under different conditions.

MATERIALS AND METHODS

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

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 bacteria were cultivated on nutrient agar at 29°C for 3 days. *B. subtilis* spores were collected following 10 days of cultivation.

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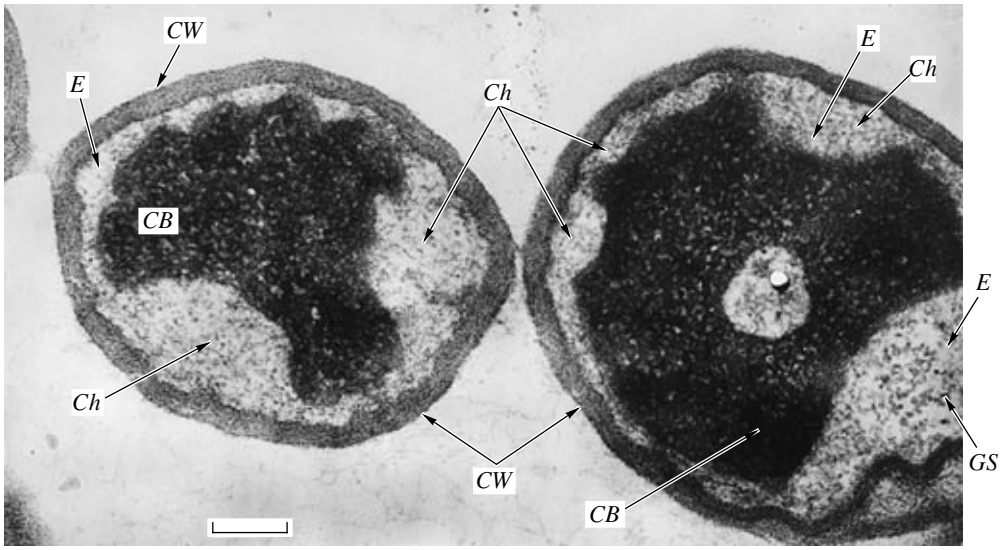

Fig. 1. Figs. 1–10. Electron microscopic images of thin sections of *S. cerevisiae* (Figs. 1–5, 8–10) and *P. pastoris* (Figs. 6, 7) cells. The thin sections were fixed and stained according to variant 1 (see the Materials and Methods section). The yeast cells were incubated in medium D at 37°C for 5 h (Figs. 1, 2), in medium D at 100°C for 5 min (Figs. 4, 5), in medium D-2 at 37°C for 5 h (Figs. 6, 7), in medium D at 37°C for 5 h followed by their treatment with micrococcal nuclease (Figs. 8, 9), and in medium D at 100°C two times for 6 min (Fig. 10). Figure 3 shows the control (untreated with chaotropic salts) cells. The scale bars represent 0.5 μm. Abbreviations used in Figs. 1–12: *CW*, cell wall; *CM*, cytoplasmic membrane; *E*, ectoplasm; *CB*, central body (endoplasm); *Ch*, chromatin; *M*, mitochondrion; *ER*, endoplasmic reticulum; *V*, vacuole; *ETS*, electron-transparent vesicular or granular structure; *GS*, ectoplasmic granular structure; *OP*, outer protein coat of spores; *IP*, inner protein coat of spores; *MS*, membrane structure; *C*, cortex.

Treatment of cells with chaotropic salts. To permeabilize cells and to extract cellular proteins and lipids, the cells were treated in three different media. D medium was 25 mM sodium citrate buffer (pH 7.0) containing 4 M guanidine thiocyanate (Fluka), 0.1 M β-mercaptoethanol, and 0.5% *N*-lauroylsarcosine (ICN Biomedicals) [1]. D-2 medium was 50 mM Tris–HCl buffer (pH 8.0) containing 4 M guanidine thiocyanate, 5 mM EDTA, 0.1 M β-mercaptoethanol, and 0.5% *N*-lauroylsarcosine. E medium was 30 mM Tris–HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride (Fluka), 5 mM EDTA, 0.1 M β-mercaptoethanol, and 0.5% *N*-lauroylsarcosine. The treatment procedure was described in detail elsewhere [5]. In this work, we used the following four variants of treatment: (a) incubation in D or D-2 medium at 37°C for 5 h with three changes of the medium; (b) incubation in D or E medium at 100°C for 5 min; (c) incubation in D or E medium at 100°C for a total of 12 min (two times for 6 min); and (d) incubation as in variant (a) followed by incubation at 37°C for 1 h in a phosphate buffer (pH 7.0) containing 1 mg/ml micrococcal nuclease (Millipore, USA). *B. subtilis* spores were treated in D-3 medium, which differed from D-2 medium by a higher concentration of EDTA (50 mM). Before treatment, D-3 medium was mixed with an equal volume of 0.1 M Tris–HCl buffer (pH 8.0) saturated with phenol. Dry spores were defatted in chloroform and suspended in water. The suspension was centrifuged, and the wet spore pellet was suspended in 10 volumes of D-3 medium. The suspension was incubated at 100°C for 10 min. Then spores were collected by centrifugation and washed twice in distilled water.

Microscopic studies were performed with an MBI-15 phase-contrast microscope and an LM-2 luminescence microscope (LOMO, Russia). In the latter case, DNA in cells was stained with a 0.4 M solution of acridine orange in a citrate–phosphate buffer (pH 4.5) or with a solution containing 1 μg/ml DAPI (Molecular Probes, Inc.).

Electron microscopic studies. Cells were harvested by centrifugation, resuspended in 0.05 M cacodylate buffer (pH 7.2) containing 1.5% glutaraldehyde, and fixed at 4°C for 1 h. The fixed cells were washed three times with the cacodylate buffer without glutaraldehyde and refixed in a 1% solution of osmium tetroxide in the cacodylate buffer at 20°C for 3 h. Further treatment was in two variants. In variant 1, the fixed cells were dehydrated in acetone and embedded in Epon 812 epoxy resin. Then the preparation was cut on an ultratome into thin sections, which were mounted on grids and contrasted with 3% uranyl acetate in 70% ethanol for 30 min and then with lead citrate [6] at 20°C for 4–5 min. Variant 2 differed from variant 1 in that the fixed cells were dehydrated in 30% ethanol containing 4% uranyl acetate and the step of lead citrate staining was omitted.

The thin sections were examined and photographed in a JEM-100B electron microscope (JEOL, Japan), which was operated at 80 kV.

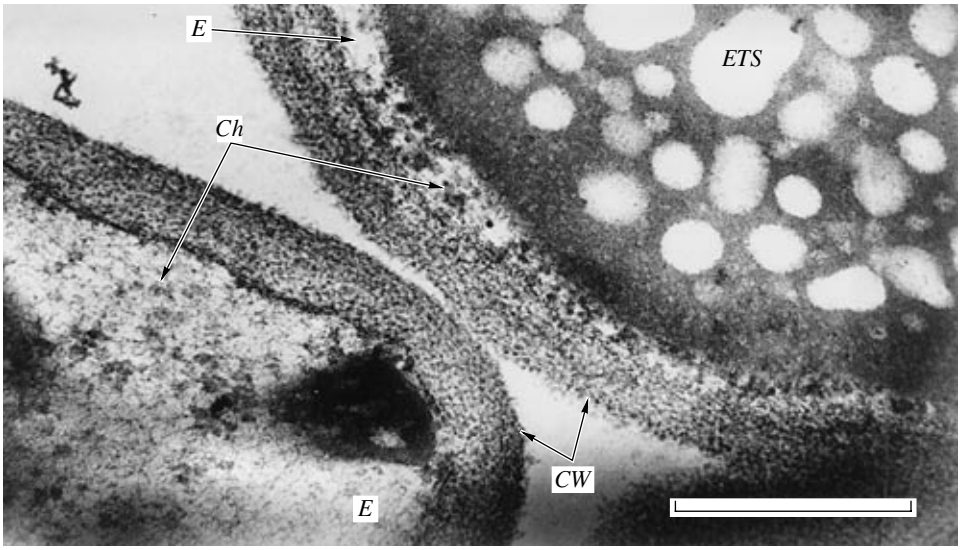

Fig. 2.

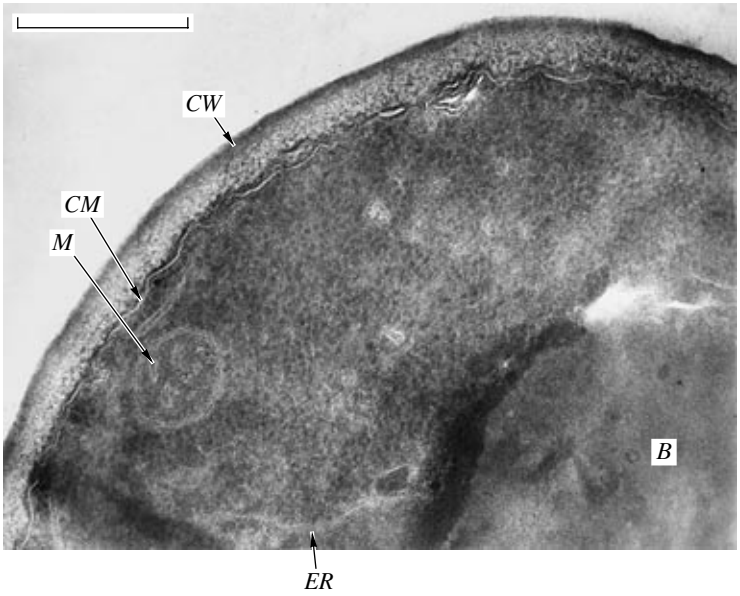

Fig. 3.

RESULTS

The electron microscopic analysis of the thin sections of the *S. cerevisiae* and *P. pastoris* cells that were treated with the chaotropic salts at 37°C for 5 h or at 100°C for 5 min showed that they underwent the following alterations:

(1) The membrane apparatus (the cytoplasmic, mitochondrial, nuclear, and vacuolar membranes and the endoplasmic reticulum) of these cells was completely degraded (Figs. 1–7, Fig. 3 representing the control). Scarce membrane fragments in the peripheral layer of modified cytoplasm (ectoplasm) (Fig. 8) had a typical trilaminar structure in cross sections and were ~75 Å thick.

(2) The ribosomes were also completely degraded and could not be identified in the remaining cytoplasm.

(3) Most of the numerous lipid granules that were present in the intact yeast cells were degraded.

(4) The protoplasm structurally differentiated into two large zones, one of which was peripheral (ectoplasm) and the other of which was central (endoplasm). The endoplasm had an electron-opaque matrix with a fine granular structure. The matrix of some cells contained either small (Figs. 1, 6, 7, 9) or large (Figs. 2, 5)

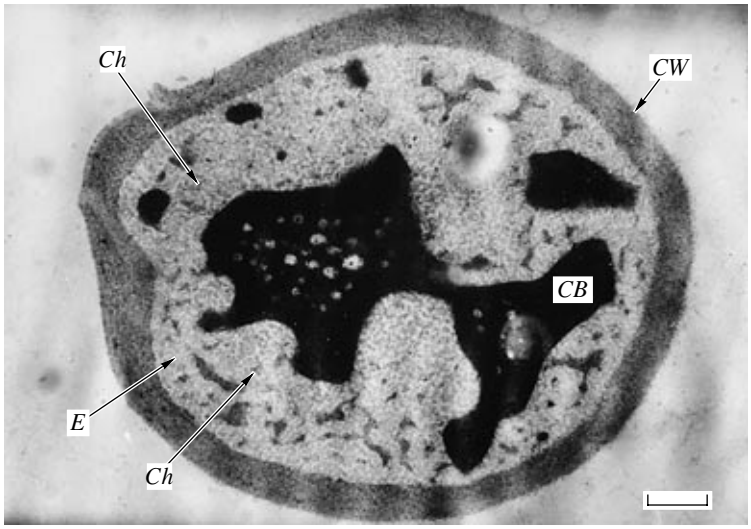

Fig. 4.

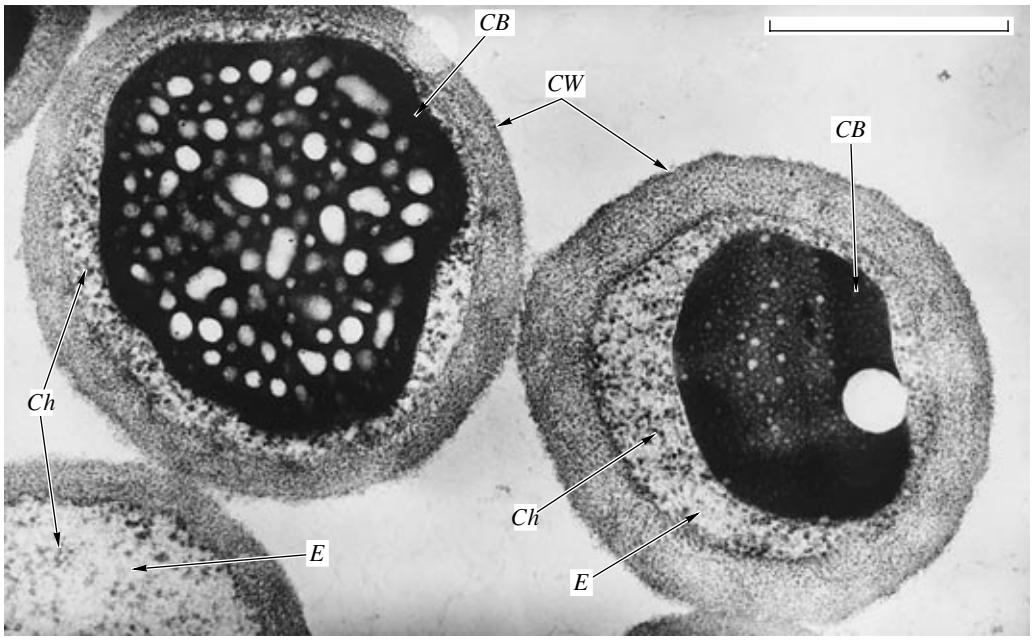

Fig. 5.

electron-transparent granular and vesicular structures, which probably resulted from the evolution of gaseous substances. The good stainability of the central body with osmium tetroxide (even after cell treatment with nucleases) (Figs. 8, 9) suggests the presence of lipids in the matrix of the central body. The ecto- and endoplasm of cells that were treated with guanidine thiocyanate and micrococcal nuclease contained short trilaminar fragments of membrane structures (Fig. 9). The small size of these fragments (~65 Å in cross sections) does not allow them to be referred to native lipid membranes. The central bodies were spheric in some cells (Figs. 5, 10) and amoeboid in others (Figs. 1, 4, 6–8) and had a diameter of 2–2.5 μm. Evaginations of the central body penetrated deep into the ectoplasm, coming sometimes into contact with the cell wall. The endoplasm did not have any envelope but was clearly separated from the ectoplasm. The latter looked like an electron-transparent layer around the central body. The thickness of this layer reached 1–1.5 μm. In *S. cerevisiae*, the reticular material of the ectoplasm contained granules ~60–80 Å in diameter (Figs. 1, 2, 4, 5). In *P. pastoris*, the fibrillar material of the ectoplasm looked like long bundles of parallel and spiral filaments

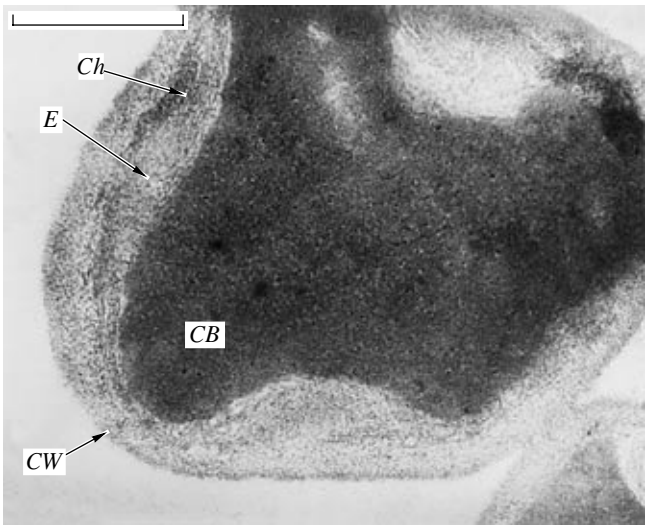

Fig. 6.

20–30 Å in diameter (Figs. 6, 7), which sometimes were circularly arranged (Fig. 7). Fibrillar structures in the ectoplasm were clearly seen only on the thin sections that were contrasted with uranyl acetate, which stains nucleic acids. These structures were similar to the DNA-containing nucleoplasm of bacteria in the size of filaments, the spiral arrangement of bundles, and stainability with uranyl acetate. Such filaments were not observed in the ectoplasm of the cells that were treated with chaotropic salts and then with micrococcal nuclease (Figs. 8, 9). These electron microscopic data suggested that the fibrillar material of the ectoplasm represents DNA. This suggestion was confirmed by the luminescence microscopic examination of the cellular location of DNA stained with acridine orange or DAPI. The degradation of membranes with chaotropic salts caused DNA to leave the cytoplasm for the peripheral zone of the protoplast. As for RNA, its location in the yeast cells treated with chaotropic salts was not comprehensively studied. The more severe treatment of yeast cells with the chaotropic salts induced the total degradation of the central body, the ectoplasm, and their components in most of the cells and led to the formation of empty cell sheaths, or cell ghosts (Fig. 10).

(5) The cell wall retained its integrity and rigidity, due to which the morphology of cells treated with chaotropic salts remained unchanged and the complexes of macromolecules and long high-polymeric DNA fibrilla, arranged in bundles and tangles, remained inside the cell ghosts, forming cell mummies (micromummies). The electron microscopic studies of the micromummies showed that their cell walls were loosened (Fig. 2) and thickened and the electron-opaque substance, which normally localizes in the surface layer of the cell wall (the mannoprotein layer), had disappeared.

As in the case of the yeast cells, the relatively mild treatment of *M. luteus* and *B. subtilis* cells with chaotropic salts also degraded their membranes, led to the formation of an ectoplasm, and caused the cellular DNA to enter and remain in the ectoplasm evaginations near the cell wall (Fig. 11), the latter retaining its ultrastructure. However, the incubation of bacterial cells in D-2 medium at 100°C for a total of 20 min (two times for 10 min) completely degraded the internal contents of the cells and converted their cell wall into separate clumps with a fairly loosened murein layer (photographs not presented). The *B. subtilis* endospores that

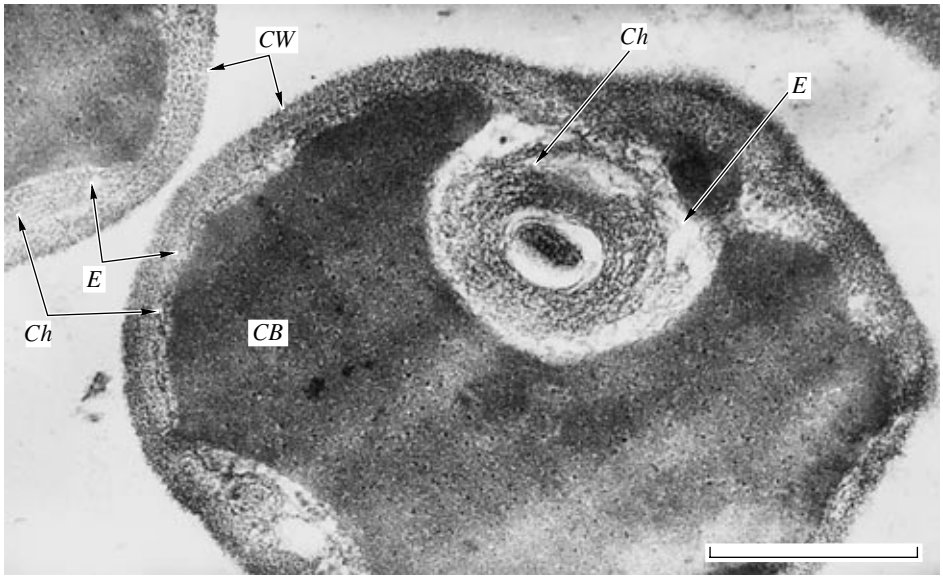

Fig. 7.

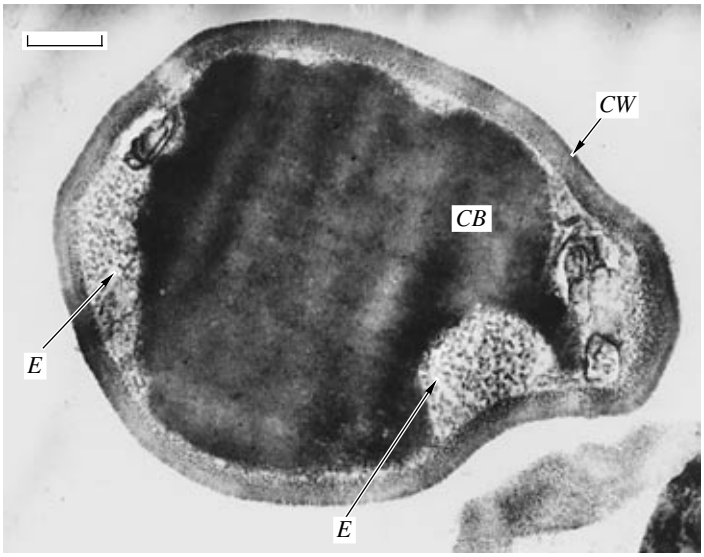

Fig. 8.

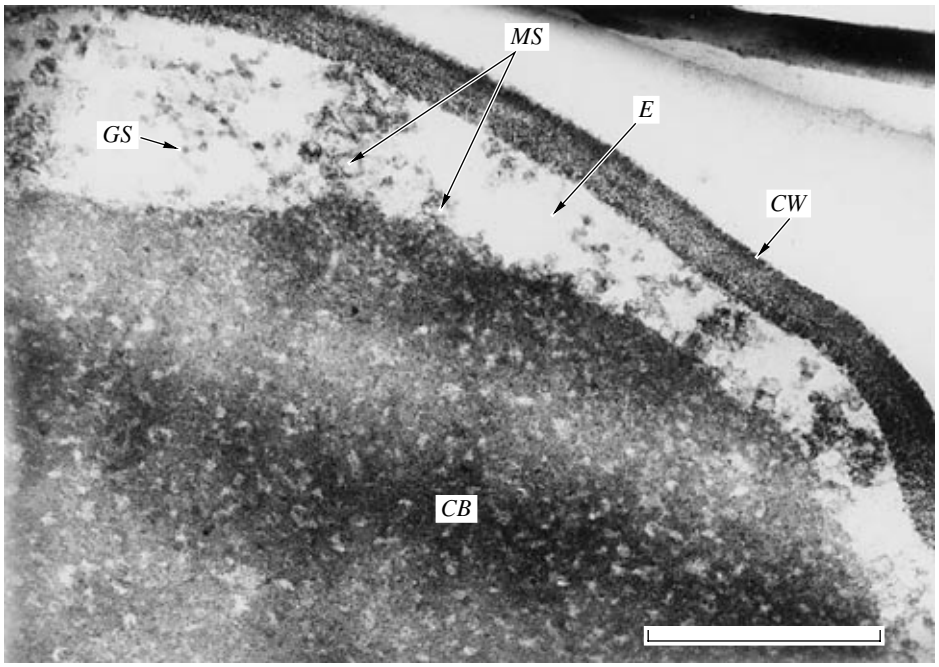

Fig. 9.

were treated with chaotropic salts lost their characteristic refractility and resistance to staining. Electron microscopic studies showed that the incubation of *B. subtilis* spores in D-3 medium with phenol at 100°C for 10 min partially degraded the outer and inner spore coats but left the cortex (the murein layer) unaltered. The spore protoplast structurally differentiated into an electron-opaque central zone and an electron-transparent peripheral zone. Cytochemical studies showed that DNA was localized in the peripheral electron-transparent zone of the spore core cytoplasm.

These data of electron and luminescence microscopy suggest that yeast cells treated with chaotropic salts in D medium at 100°C for 5 min and bacterial cells treated with chaotropic salts in D-2 medium at 100°C for 5 min are suitable for PCR analysis in situ. The genomic DNA–containing mummies of the vegetative *M. luteus* and *B. subtilis* cells and *B. subtilis* spores, which were obtained by treating them with chaotropic salts, were subjected to PCR analysis with the universal primers 21 and 15/19 [5]. This analysis showed that the mummies gave rise to different DNA fragments, which

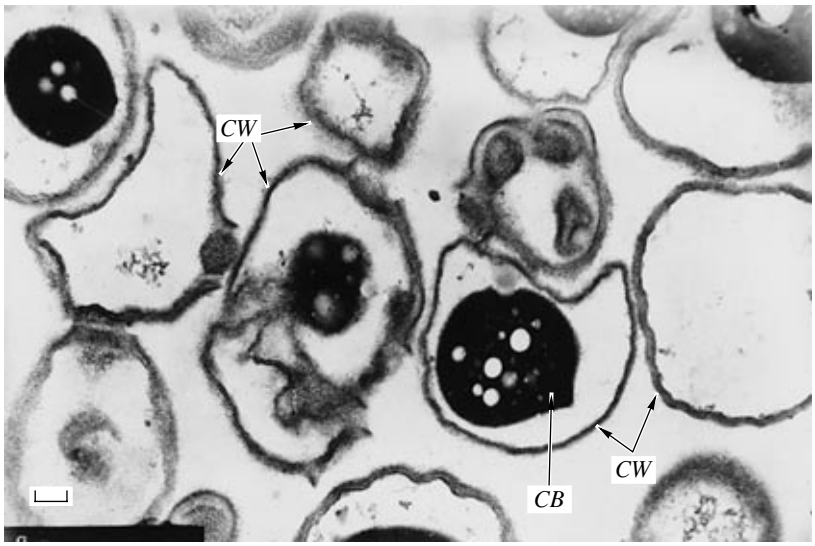

Fig. 10.

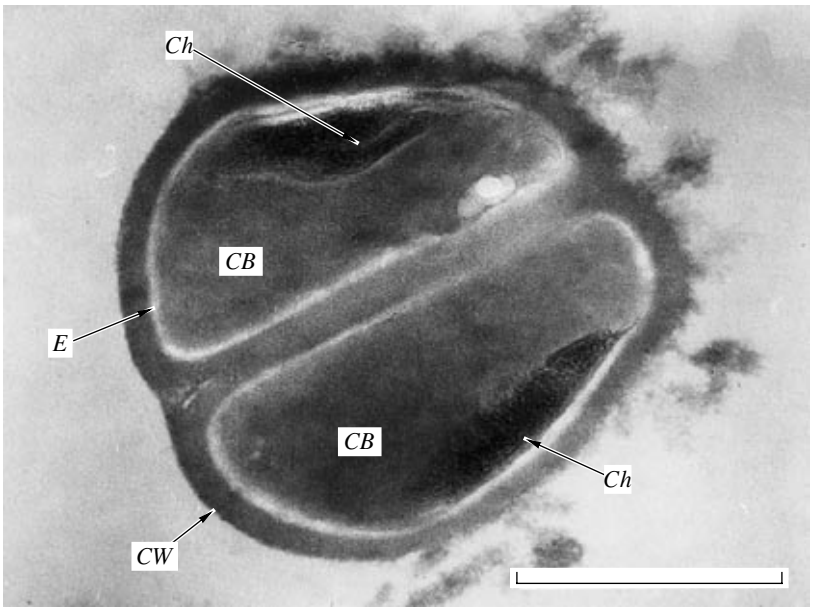

Fig. 11. Electron microscopic image of a thin section of *M. luteus* cells incubated in medium D-2 at 100°C for 5 min. The thin section was fixed and stained according to variant 2 (see the Materials and Methods section). The bar represents 0.3 μm.

were specific to the microorganisms from which the mummies were obtained. Consequently, the cells and spores of gram-positive bacteria treated with chaotropic salts are able to release DNA into the reaction mixture during thermal PCR cycles.

The electron microscopic studies of the fine structure of yeast and bacterial cells that were treated with chaotropic salts allow two important inferences to be drawn: (1) chaotropic salts separate genomic DNA from other cellular constituents (proteins and lipids) and cause it to occupy a peripheral position and (2) chaotropic salts loosen the cell wall, making it permeable to exogenous proteins. As a result, the genomic DNA of micromummies (formerly, DNA-containing cell envelopes) can serve as a template DNA in polymerase chain reactions, which occur both inside and outside the micromummies.

DISCUSSION

The treatment of live microbial cells with chaotropic salts transforms them to micromummies, which consist of the cell wall and remnants of the cytoplasm, DNA, and RNA enclosed in cell sheaths (Figs. 1–12). The transformed remnants of the cytoplasm in the endoplasm are

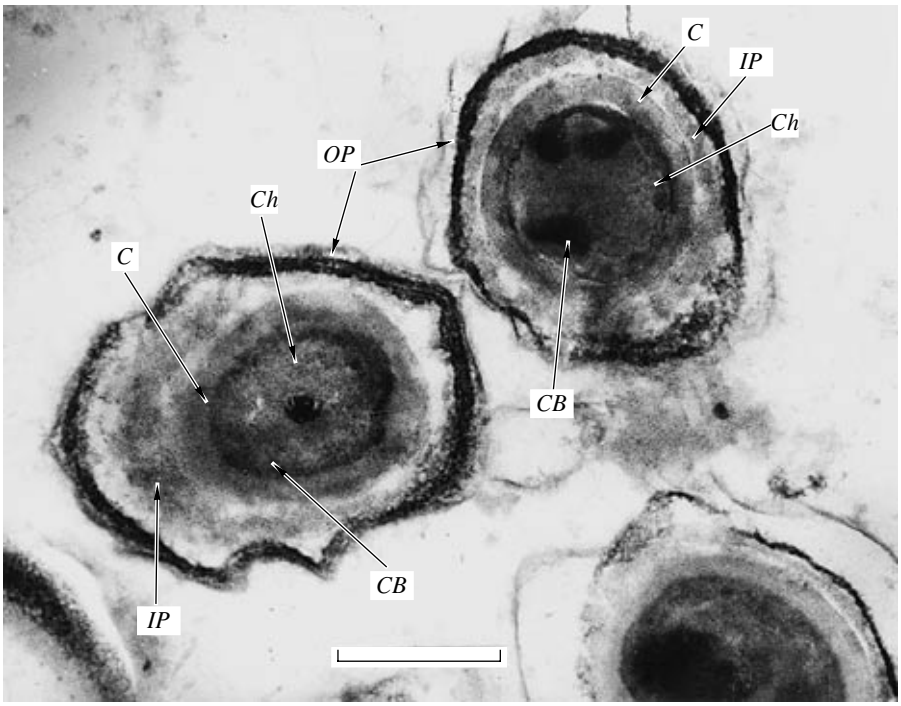

Fig. 12. Electron microscopic image of a thin section of *B. subtilis* spores incubated in medium D-2 at 100°C for 7 min. The thin section was fixed and stained according to variant 1 (see the Materials and Methods section). The bar represents 0.3 μm.

resistant to the chaotropic salts (at least under the treatment conditions employed). The conservation of the cytoplasm, DNA, and RNA is due to enzyme inactivation by the chaotropic salts and, as a consequence, the absence of autolytic processes. The micromummies induced by the chaotropic salts are structurally similar to the micromummies that were produced with the aid of alkylhydroxybenzenes (AHBs), 4-*n*-hexylresorcinol in particular [7]. Like the chaotropic salts, the AHBs completely degrade cell membranes but are unable to induce the formation of ectoplasm that would contain native DNA.

The micromummies of *S. cerevisiae* and *P. pastoris* cells induced by their treatment with chaotropic salts at 37–40°C do not differ considerably from the micromummies that are induced by treatment at 100°C for 5 min. In both cases, the cytoplasm structurally differentiates into two large zones, one of which is peripheral and electron-transparent (ectoplasm) and the other of which is central and electron-opaque (endoplasm). The presence of DNA in the ectoplasm and its low electron density suggest that the ectoplasm is hydrophilic. Conversely, the presence of lipids in the endoplasm implies that this zone is hydrophobic. RNA, which is hydrophilic like DNA, must localize in the ectoplasm also. As for the cellular location of proteins, it must depend on their hydrophobicity/hydrophilicity balance. Numerous hydrophobic proteins (first of all, membrane proteins) must localize, together with lipids, in the endoplasm. Hydrophilic proteins, most of which are released from cells during their treatment with chaotropic salts, must localize in the ectoplasm. It should, however, be noted that chaotropic salts can bring about the denaturation of hydrophilic proteins and thus change their conformation and expose the hydrophobic interior to the outside, causing these proteins to become superficially hydrophobic. The good staining of the endoplasm with lead citrate shows that most proteins, indeed, localize in the endoplasm of micromummies. The location of genomic DNA in the hydrophilic ectoplasm near the loose (and, hence, permeable) cell wall of prokaryotic and eukaryotic microorganisms explains why such DNA is cleaved by exogenous nucleases and why the hydrophobic components of the endoplasm do not protect DNA from the nucleases.

Microscopic studies of the effect of chaotropic salts on microbial cells are necessary to control this process cytologically and, together with biochemical studies, to reveal the structural and functional aspects of PCR inside cells (PCR in situ) and to choose optimal conditions for the PCR. PCR in situ is of great importance for the taxonomic identification of microorganisms in natural substrates at species and group levels. The current methods of PCR in situ are, however, intricate and poorly reproducible [9]. Experiments with fluorescent oligonucleotide primers to the yeast 18S rRNA genes provide evidence that PCR can occur inside yeast cells that have been treated with chaotropic salts, with the fluorescent reaction product remaining in the cells (data not presented). This approach can probably be employed for the in situ PCR analysis of other groups

of microorganisms, gram-positive bacteria in particular. This probability should be proved experimentally.

ACKNOWLEDGMENTS

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