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EXPERIMENTAL ARTICLES

# Obtaining and Characterization of DNA-Containing Micromummies of Yeasts and gram-Positive Bacteria with Enhanced Cell Wall Permeability: Application in PCR

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**Abstract**—The procedure of obtaining DNA-containing cell envelopes ("micromummies") of bacteria, yeasts, and fungi using chaotropic salts has been developed previously and the possibility of their direct application in PCR has been demonstrated. The fine structure of micromummies has been studied by electron microscopic methods. This work has demonstrated that additional treatment of micromummies of yeasts and gram-positive bacteria with proteinase K results in hydrolytic degradation of cell proteins and drastic enhancement of cell wall permeability for macromolecules (DNA). Thus, the efficiency of PCR ex situ using resultant micromummies after washing off the products of protein hydrolysis and proteinase K can be increased. The results of electron microscopic study of ultrathin sections of yeasts (*Pichia pastoris, Saccharomyces cerevisiae*) and gram-positive bacteria (*Micrococcus luteus, Arthrobacter globiformis, Bacillus subtilis*) support the biochemical data that treatment with chaotropic salts and proteinase K results in the loosening of microbial cell walls and in a decrease in the intracellular protein content. At the same time, cell walls generally maintain their integrity (continuity) and initial spherical or rodlike shape. The optimal modes of treatment of the cells of different microbial species with chaotropic salts and proteinase K have been selected to obtain permeabilized cell envelopes containing denatured or native DNA.

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*Key words*: ultrastructure of yeasts and gram-positive bacteria, chaotropic salts, proteinase K, DNA-containing cell envelopes, PCR amplification.

Yeasts, filamentous fungi, and gram-positive bacteria are known to have thick and strong cell walls resistant to most lytic agents. In contrast to gracilicute cells of gram-negative bacteria, which are easily destroyed by a number of physical and chemical factors, the cell walls of fungi and gram-positive bacteria endure treatment with solutions of detergents, alkali, and chaotropic mixtures. This fact strongly impedes DNA isolation from such organisms.

The procedure of rapid preparation of DNA samples from yeasts and filamentous fungi for PCR amplification has therefore been developed. The approach applied includes the treatment of cells of the above microorganisms with buffer solutions containing chaotropic salts (4 M guanidinium thiocyanate or 6 M guanidinium chloride) [1].

Chaotropic salts (CS) have a specific effect on living microbial cells: they destroy membranes and other cell structures, initiate quick denaturation of all the cell proteins including nucleases, thus causing DNA conversion into a free form [1–5]. As a result, it has been proposed previously to use some chaotropic salts for DNA and RNA isolation from gram-negative bacterial cells and from animal cells and tissues [6–9].

As we have revealed earlier, the treatment of yeast and gram-positive bacterial cells with chaotropic solutions within a wide temperature range (20 to 100°C) results in cell wall permeabilization and makes it possible to extract a considerable portion of proteins, lipids, RNA, and low-molecular components from the cells. At the same time, the cell walls of these microorganisms are not degraded completely and maintain their shape even after boiling in CS solutions, with essentially all genomic DNA retained in CS-treated cells [1, 2, 10].

CS treatment in the temperature range of 20 to 40°C for several hours yields "cell envelopes" (CE) of yeasts and gram-positive bacteria with intact genomic DNA.

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If the cells are treated with CS at 100°C for several minutes, the chromosomal DNA is denatured and partially fragmented; however, even in this case nearly all of it remains inside the CE [1, 2, 10]. Such DNA easily diffuses into solution and may be used as a PCR template [1]. It should be noted that DNA as a component of CE is accessible for nonspecific (micrococcal nuclease) and specific (restriction endonucleases) nucleases [1].

The electron microscopic analysis of ultrathin sections of the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* showed that CS treatment of cells at 37°C for 5 h or at 100°C for 5 min resulted in complete degradation of the membrane apparatus, degradation of ribosomes, and destruction of most of the lipid granules. In addition, the state of the protoplasm drastically changes as it is differentiated into two large zones: the peripheral electron-transparent zone (ectoplasm) and the central electron-dense zone (endoplasm). At the same time, DNA is localized as fibrils in the ectoplasm, i.e., on the periphery. The endoplasm consists of hydrophobic proteins and lipids [2]. Thus, the electron microscopy data demonstrate that, contrary to initial assumptions [1], CS-treated cells retain a considerable part of their proteins and lipids (as lipid granules).

The following changes are noted in the yeast cell-wall ultrastructure: loosening of the cell wall (CW) matter, increase of CW thickness, and disappearance of the electron dense substance localized in the surface part of the CW (mannoprotein layer) [2]. In the bacteria *Bacillus subtilis* and *Micrococcus luteus*, the effect of CS also induces degradation of membranes, formation of ectoplasm, and release of DNA into this layer; CW in this case remain completely intact and do not undergo any noticeable ultrastructural changes [2]. We have termed these structurally transformed cells "micromummies" (MM) [2, 10].

The developed procedure of obtaining DNA-containing MM proved to be efficient for the yeasts and filamentous fungi isolated from nature (environmental isolates) [1] and for the yeasst isolated from clinical samp-les (representatives of the genera *Candida, Rhodotorula, Trichosporon*, and *Malassezia*) (unpublished data). However, in our experiments natural yeast isolates with an extremely dense cell wall occurred, which were therefore poorly amenable to the action of CS even after boiling in a water bath for 10 min. The treatment of such cells by chaotropic solutions at 37°C was completely inefficient.

As for gram-positive bacteria, some representatives of this group were found to have a rather fast cell wall and to endure the CS treatment at 100°C for 5–7 min (clinical isolates of *Staphylococcus aureus*, environmental strains of S*treptococcus thermophilus*, strains of *Corynebacterium ammoniagenes*, etc.). We have obtained DNA-containing CE of these bacteria suitable for direct use for PCR amplification (unpublished data). On the other hand, cell walls of some laboratory strains of bacilli (e.g., *Bacillus subtilis* 168, *Bacillus amyloliquefaciens*) do not endure even 5 min of boiling in the buffer with 4 M guanidinium thiocyanate and undergo lysis under these conditions. The procedure of DNA isolation from such strains presents no difficulties.

We faced considerable difficulties in the work with soil isolates (strains) of gram-positive bacteria: *Bacillus subtilis*, *Arthrobacter globiformis*, and *Micrococcus luteus*. The analyzed bacteria had very fast and dense cell walls, which prevented almost completely the release of intracellular proteins in the course of treatment with chaotropic solutions. To obtain the MM of these bacteria, it was necessary to increase the duration of boiling of appropriate cells in the buffer with 4 M guanidinium thiocyanate to 20 min. However, even under these conditions of CS treatment, most of the protein still remained inside the cells, and the PCR procedure with the MM obtained under these conditions included 35–37 cycles (with the norm of 29–31 cycles) [2].

Thus, the problem was to modify (improve) the previously developed procedure of obtaining DNA-containing MM of yeasts and gram-positive bacteria. This problem was solved by a new methodical approach that consisted in successive treatment of cells first with CS and then with proteinase K (PK). Both CS and PK are used separately for DNA isolation from soil [11] and for FISH [12] and PCR [13] reactions. However, their combined use has never been studied and the structural bases of FISH and PCR reactions with model microorganisms have not been elucidated.

## MATERIALS AND METHODS

**Reagents** used included: guanidinium thiocyanate (Fluka, Switzerland), sarcosyl (lauroylsarcosyl, Na salt) (ICN Biomedicals, United States), SDS (sodium dodecyl sulfate) (Serva, United States), components of nutrient media (Difco, United States), and proteinase K (Boeringer Mannheim, Germany). Thermostable *Taq* polymerase was provided by L.I. Patrushev (Institute of Bioorganic Chemistry, Russian Academy of Sciences). Molecular weight markers of DNA fragments were a 1 kb DNA ladder (Fermentas, Lithuania). Other reagents were from Sigma (United States).

**Strains of the yeasts and gram-positive bacteria.** The following microbial cultures were used in the work: yeasts *Saccharomyces cerevisiae* (Y190, Institute of Bioorganic Chemistry, Russian Academy of Sciences) and *Pichia pastoris* (GS115, Institute of Bioorganic Chemistry, Russian Academy of Sciences) and gram-positive bacteria *Arthrobacter globiformis* (VKM B-661), *Bacilus subtilis* (VKM B-504), and *Micrococcus luteus* (NCIMB 13267).

**Yeast cells were grown** at 30°C in a liquid YPD medium (1.0% yeast extract, 2.0% bacto peptone, 2.0% glucose, pH 5.3) or on plates with agarized YPD medium (1.5% agar). The cells of gram-positive bacteria were grown on plates with L-agar [14] at 37°C.

**Buffer solutions with chaotropic salts:** cells were treated with buffer D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.1 M β-mercaptoethanol, 0.5% sarcosyl) [6] or buffer D-2 (4 M guanidinium thiocyanate, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1 M β-mercaptoethanol, 0.5% sarcosyl) [1].

**Obtaining of "DNA-containing cell envelopes" (MM); treatment of cells with chaotropic solutions and proteinase K.** The cells of yeasts (30–50 mg wet biomass) or gram-positive bacteria (ca. 30 mg of biomass) were suspended in 400–500 μl of buffer D or D-2 and incubated in a water bath for 5–10 min. The cells were precipitated by centrifugation and the supernatant was removed. Cell pellets were washed twice in 1 ml of distilled (deionized) water. After the second wash, cell pellets were resuspended in the buffer containing: 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% sarcosyl (or 0.3% SDS), supplemented with PK (50 μg/ml). The mixtures with proteinase were incubated for 0.5–2.5 h at 37°C and then centrifuged. The supernatant was collected into clean tubes; the pellets were washed twice with 1 ml of distilled water and then suspended in 300 μl of 50% ethanol. Suspensions of DNA-containing cell envelopes were stored at 4°C.

**The assay of protein and nucleic acids in supernatants after the treatment of micromummies with proteinase K.** All the supernatants obtained by the treatment of each cell envelopes sample with proteinase K and subsequent washing were combined (2.5 ml total), and absorption in the total solution was measured at 260 and 280 nm in order to assess the quantity of extracted proteins (peptides). Protein concentration was determined according to the equation: C (mg/ml) = $1.55E_{280} - 0.76E_{260}$ [15].

To study the release of nucleic acids (NA) from the cells during the treatment with proteinase K and water washings, aliquots were taken from combined supernatants (generally 0.5 of 2.5 ml) with the addition of two volumes of 96% ethanol. The mixture was centrifuged, and the resulting NA-containing pellets were air-dried and dissolved in 50 μl of distilled $H_2O$. The presence of NA in the resulting solutions was tested electrophoretically. For electrophoresis, 10 μl of solution was placed into each gel hole.

**PCR amplification with gene-specific primers.** In all cases, PCR was performed using an MJ Research PTC-200 thermocycler. The reaction mixture (50 μl) contained PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1.5–2.0 mM $MgCl_2$, a mixture of four dNTP (0.2 mM each), primer oligonucleotides (10–15 pmol each), 1 μl of the suspension of DNA-containing cell envelopes (or 1–10 ng of purified yeast (bacterial) DNA), and 2.5 activity units of thermostable *Taq* polymerase. Each PCR experiment had a negative control: a sample without template DNA.

PCR amplification of ribosomal DNA fragments in different yeast strains was carried out using primers 5.8S-R: (5') TCGATGAAGAACGCAAGC, and LR3: (5') GGTCCGTGTTTCAAGAC [16]. The PCR conditions were as follows: denaturing, 93°C, 30 s; annealing, 55°C, 30 s; synthesis, 72°C, 40 s. The number of cycles: 30–40. PCR amplification of ribosomal DNA fragments in eubacteria was carried out using primers 27f: (5') AGAGTTTGATCCTGGCTCAG, and 1522r: (5') AAGGAGGTGATCCARCCGCA [17]. The PCR conditions were as follows: denaturing, 93°C, 30 s; annealing, 60°C, 30 s; synthesis, 72°C, 60 s. The number of cycles: 30–40.

**Determination of PCR sensitivity with yeast and bacterial CE.** The sensitivity of PCR with DNA-containing cell envelopes was determined using serial dilutions ($10^{-1}$, $10^{-2}$ … $10^{-8}$) of the corresponding CE suspensions in distilled water. One μl of each dilution was added to the PCR reaction mixture containing primers 27f + 1522r or LR3 + 5.8S-R. The number of PCR cycles varied from 30 to 40 depending on the dilution of the initial suspension. The PCR was performed as described above. The presence (absence) of DNA fragments (PCR products) was assayed electrophoretically.

**PCR products and other NA-containing solutions were analyzed** by electrophoresis in 1% agarose gel using standard Tris-acetate buffer with ethidium bromide [18].

**Light microscopy studies** were carried out using the microscopes MBI-15U equipped with a phase contrast device and LUMAM-2 (LOMO) in the epifluorescent mode. Fluorochrome staining of cellular DNA was performed with diamidino-2-phenylindole (DAPI, Molecular probes); a solution of 2 μg/ml DAPI in phosphate buffer (pH 7.0) was used.

**Electron microscopic methods of study.** The pellet of microbial cells after centrifugation was fixed in 1.5% solution of glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4°C for 1 h, washed three times in the same buffer, and additionally fixed in 1% $OsO_4$ solution in 0.05 M cacodylate buffer (pH 7.2) for 3 h at 20°C. After dehydration, the material was embedded in Epon 812. Ultrathin sections were mounted on support nets and contrasted for 30 min with 3% uranyl acetate solution in 70% alcohol and additionally stained with lead citrate according to Reynolds at 20°C for 4–5 min. Ultrathin sections were examined and photographed in an JEM-100B electron microscope (JEOL, Japan) at the accelerating voltage of 80 kV.

## RESULTS AND DISCUSSION

In accordance with the data obtained previously, cell envelopes of the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* after CS treatment (at 37 and 100°C) became permeable for RNase A, micrococcal nuclease, and even restriction nucleases [1]. In view of these data, we have decided to use proteinase K for the degradation and complete removal of denatured proteins from the micromummies. With regard to the fact that both yeast and bacterial cell walls comprise a particular group of

proteins [18, 19], one could also anticipate changes in cell wall properties after the action of PK.

In the experiment, cells of the yeasts *S. cerevisiae* and *P. pastoris* (approximately 50 μl of wet biomass) were suspended in buffer D and the suspension was boiled for 7 min; then the cells were precipitated by centrifugation and washed with distilled water. The cells of gram-positive bacteria *Arthrobacter globiformis*, *Bacillus subtilis* and *Micrococcus luteus* (approximately 30 μl of wet biomass) were also suspended in buffer D and boiled in a water bath for 10 min. In parallel, the cells of the yeasts and gram-positive bacteria were treated with buffer D at 37°C for 15 h. After appropriate washing, the obtained DNA-containing cell envelopes were transferred to the buffer with proteinase K and incubated with the enzyme for 2.5 h at 37°C. After that, cells were precipitated by centrifugation and washed twice with distilled water. The supernatants were tested for the presence of DNA and RNA, and the quantity of extracted proteins (peptides) was determined.

According to the spectrophotometry data (absorption at 260 and 280 nm), the combined supernatants contained a significant amount of proteins (peptides) (table). The maximal release of protein was observed in the variant with *B. subtilis* cells (independent of the temperature of CS treatment: 37°C or 100°C).

The presence of NA in combined supernatants was examined as well. Electrophoregrams of the corresponding NA samples are presented in Fig. 1. It can be seen that genomic DNA was completely absent (not detected) in the supernatants of MM samples from *A. globiformis*, *M. luteus*, and *P. pastoris* obtained by boiling with CS in a water bath for 10 min. The results indicate that genomic DNA, denatured after boiling with CS, practically does not dissipate (is not released from the cells) in the course of the subsequent treatment of MM with proteinase K and washing with distilled water. Previously we have suggested that DNA was present in the interior of cell envelopes as a three-dimensional net [1]. Apparently, this net is not destroyed at 20–37°C. *B. subtilis* cells were an exception. Their supernatants contained high-molecular DNA (see Fig. 1, lane *6*); most of it remained at the start during electrophoresis. The reason for such abnormal behavior of *B. subtilis* cells will be considered below.

At the same time, the data presented in Fig. 1 demonstrate that combined supernatants corresponding to the MM of gram-positive bacteria (except for *B. subtilis*) and the yeast *P. pastoris* obtained using buffer D at 37°C contain a minor amount of high-molecular DNA (according to our estimates, less than 1% of the whole genomic DNA). This finding means that the loss of native genomic DNA from the MM of yeasts and gram-positive bacteria in the course of their treatment with proteinase K is insignificant, and may be neglected. The data presented in Fig. 1 (lanes *2, 6*) also show that, in the case of *B. subtilis* cells, the supernatant contains not only DNA but also a noticeable quantity of RNA.

The release of proteins (peptides) after the action of proteinase K on DNA-containing cell envelopes of microorganisms

| CE (micromummies) of microorganisms | Conditions of obtaining CE (micromummies) | Absorption in supernatants $A_{260}$ | $A_{280}$ | Total protein (mg) in supernatants |
|---|---|---|---|---|
| *A. globiformis* | Buffer D, 100°C, 10 min, PK, 2.5 h | 0.674 | 1.101 | 1.195 |
| *B. subtilis* | idem | 3.505 | 2.615 | 1.389 |
| *M. luteus* | idem | 0.917 | 1.420 | 1.504 |
| *P. pastoris* | Buffer D, 100°C, 7 min, PK, 2.5 h | 0.930 | 1.032 | 0.894 |
| *A. globiformis* | Buffer D, 37°C, 15 h, PK, 2.5 h | 0.324 | 0.630 | 0.730 |
| *B. subtilis* | idem | 4.074 | 2.850 | 1.321 |
| *M. luteus* | idem | 0.651 | 1.023 | 1.091 |
| *P. pastoris* | idem | 0.470 | 0.725 | 0.767 |

Note: Protein concentration was assayed according to the equation: C (mg/ml) = $1.55E_{280} - 0.76E_{260}$ [15].

The MM obtained with proteinase K and containing denatured and native DNA were further used for PCR with universal gene-specific primers to the genes of ribosomal RNA. The results of electrophoresis in 1% agarose gel of the DNA fragments (PCR products) obtained using the MM of gram-positive bacteria with denatured DNA as template and primers 27f + 1522r are presented in Fig. 2a. It can be seen that DNA fragments of the same size (approx. 1500 bp) are formed in all three variants in the course of PCR. At the same

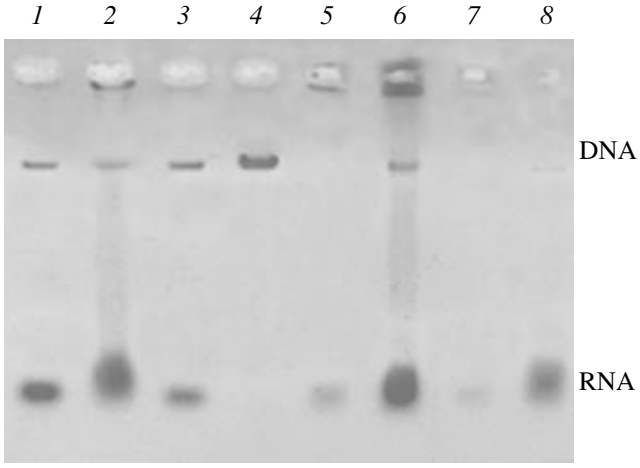


**Fig. 1.** Electrophoretic analysis of NA in the supernatants after the treatment of cell envelopes (CE) of *A. globiformis* (*1, 5*), *B. subtilis* (*2, 6*), *M. luteus* (*3, 7*), and *P. pastoris* (*4, 8*) with proteinase K. Lanes *1–4* correspond to CE obtained at 37°C for 15 h; lanes *5–8* correspond to CE obtained at 100°C for 10 min.

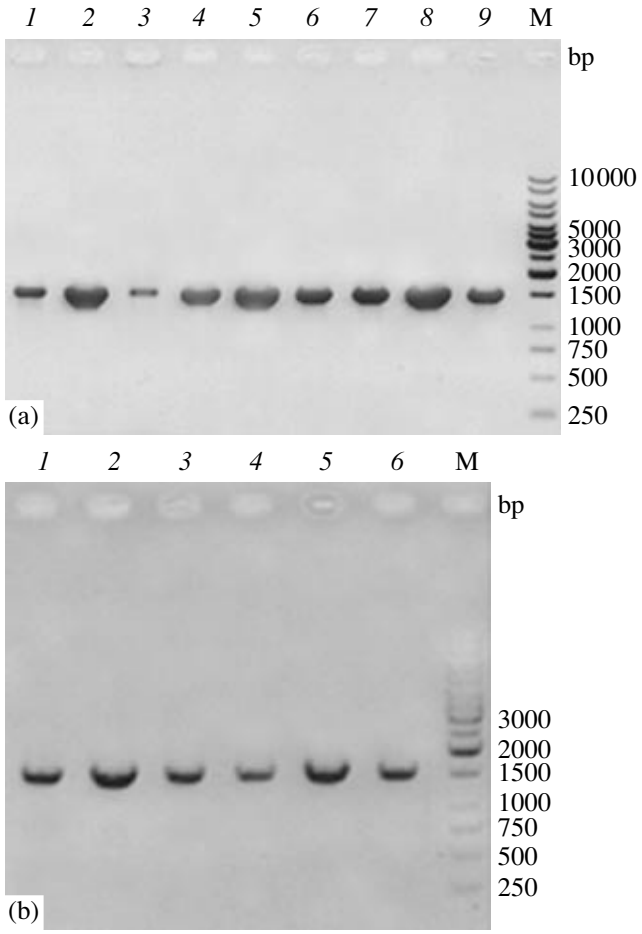


**Fig. 2.** Electrophoretic analysis of the products of PCR amplification (30 thermal cycles) of ribosomal DNA fragments of three species of gram-positive bacteria using primers 27f + 1522r. The template was obtained from DNA-containing cell envelopes of *A. globiformis* (*1, 4, 7*), *B. subtilis* (*2, 5, 8*), and *M. luteus* (*3, 6, 9*) after successive treatment of bacteria: (a), in buffer D at 37°C for 10 min and then with proteinase K; (b), in buffer D at 37°C for 15 h and then with proteinase K. Bacterial CE were taken from different dilutions (*1, 2, 3*, from $10^{-4}$; *4, 5, 6*, from $10^{-2}$; *7, 8, 9*, from $10^{-3}$). Length markers of DNA fragments (M).

time, the yield of PCR products (judging by the intensity of the bands on the electrophoregram) does not change significantly depending on the amount of the template, i.e., cell envelopes added to the reaction mixture. Obviously, there is an apparent excess of the template in this case (MM suspension from tenfold dilutions was added in 1 μl amounts to 50 μl of reaction mixture; the number of PCR cycles in this experiment was 30). In a separate experiment, it was established that the number of cycles could be reduced to 27, all other factors being the same, with fairly distinct bands on electrophoregrams. It should be noted that the treatment of the same bacteria in buffer D at 100°C (but without proteinase K) for 10 min was obviously insufficient to obtain a PCR product, even with 40 thermal cycles.

Similar results were obtained with the MM of three bacterial strains containing native DNA. Without the treatment with proteinase K, the mummies of these gram-positive bacterial cells were absolutely unable to promote the formation of PCR products. At the same time, the MM formed after the treatment with proteinase K were very effective as PCR template (see Fig. 2b).

Similar results were obtained with the MM of the yeasts *S. cerevisiae* and *P. pastoris*. The MM of both strains under study, containing both denatured and native DNA, were found to be equally effective as PCR templates. In the presence of primers 5.8S-R and LR3 and the corresponding MM (i.e., DNA template), amplification of the intergenic region (spacer) IST2 and region D1/D2 of the 26S subunit of rDNA occurs [16]. The sizes of the PCR products for *S. cerevisiae* and *P. pastoris* strains in our experiment were 900 and 500 bp, respectively, which correlated well with the previous results [1]. It should be mentioned that previously we had failed to obtain PCR products with the yeast MM containing native DNA.

The phenomenon of retaining denatured DNA in cell envelopes (micromummies) after their treatment with proteinase K and washing with water has been studied on other yeast strains as well. For example, we have obtained the CE of three clinical yeast isolates from the genera *Candida, Rhodotorula*, and *Trichosporon* (biomass of the three strains was kindly provided by V.G. Arzumanyan, Mechnikov Research Institute of Vaccines and Serums, Russian Academy of Medical Sciences). They were obtained by boiling of the cells in buffer D for 6 min. The resulting DNA-containing CEs of analyzed yeasts were studied by PCR with primers 5.8S-R and LR3. They were found not to differ in their characteristics from the CEs of *S. cerevisiae* and *P. pastoris*. The CEs of three clinical isolates were stored in 50% ethanol for more than a year at 4°C. Then they were placed into an appropriate buffer with 0.3% SDS and treated for 1 h with proteinase K (50 μg/ml). We determined protein concentrations and the presence of DNA and RNA in combined supernatants after washing off proteinase K.

Electrophoregrams showed no DNA in the supernatants. In other words, denatured DNA was not washed out of CE. On the contrary, the combined supernatants contained a quantity of proteins comparable with that found in the CE of *S. cerevisiae* and *P. pastoris* (data not shown).

Similar results were also obtained in the work with 10 natural isolates of psychrophilic yeast of the genus *Leucosporidium* (the biomass of these strains was provided by G.A. Lisichkina, Faculty of Soil Science, Moscow State University). The cell envelopes of *Leucosporidium* sps. obtained by successive treatment with chaotropic salts (100°C, 6 min) and proteinase K (1 h, 37°C) did not lose their DNA and were highly effective sources of templates for PCR amplification (data not shown).

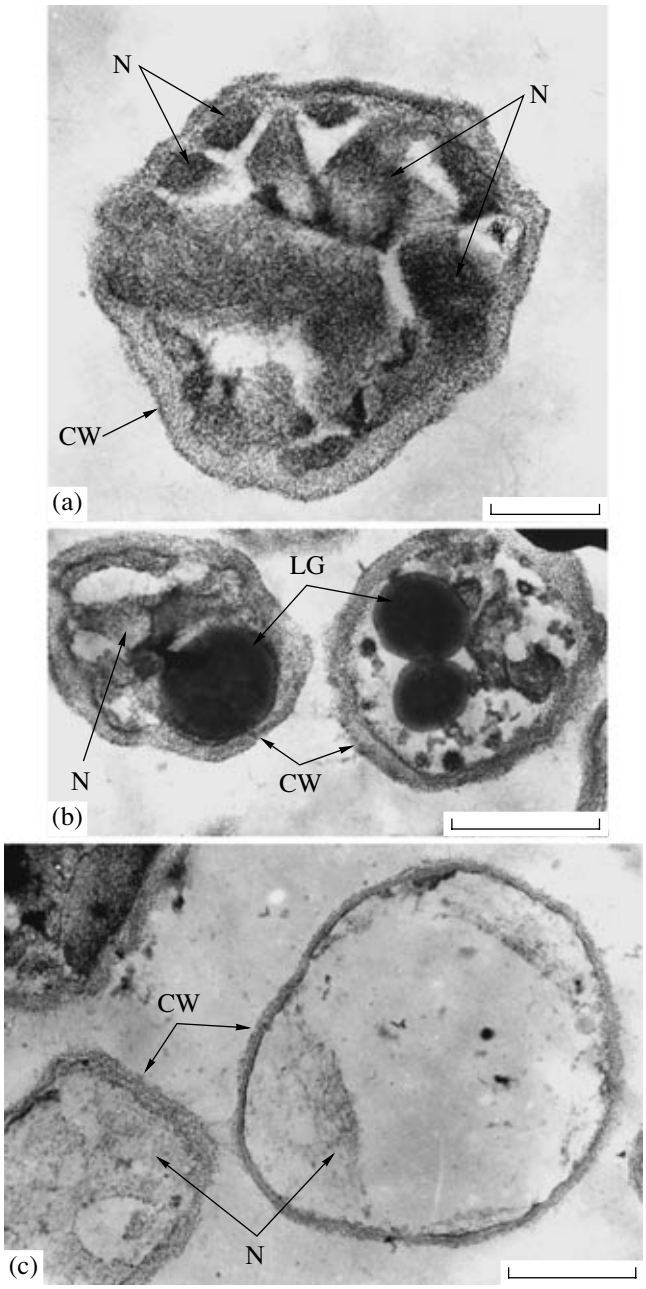


**Fig. 3.** Ultrathin sections of *P. pastoris* cells: (a) (b) treated in buffer D at 37°C for 15 h and then with proteinase K; (c) treated in buffer D at 100°C for 7 min and then with proteinase K. The scale bar in Fig. 3a is 0.5 μm, in Fig. 3b and 3c, 1 μm. CW, cell wall; N, nucleic substance; LG, lipid granules.

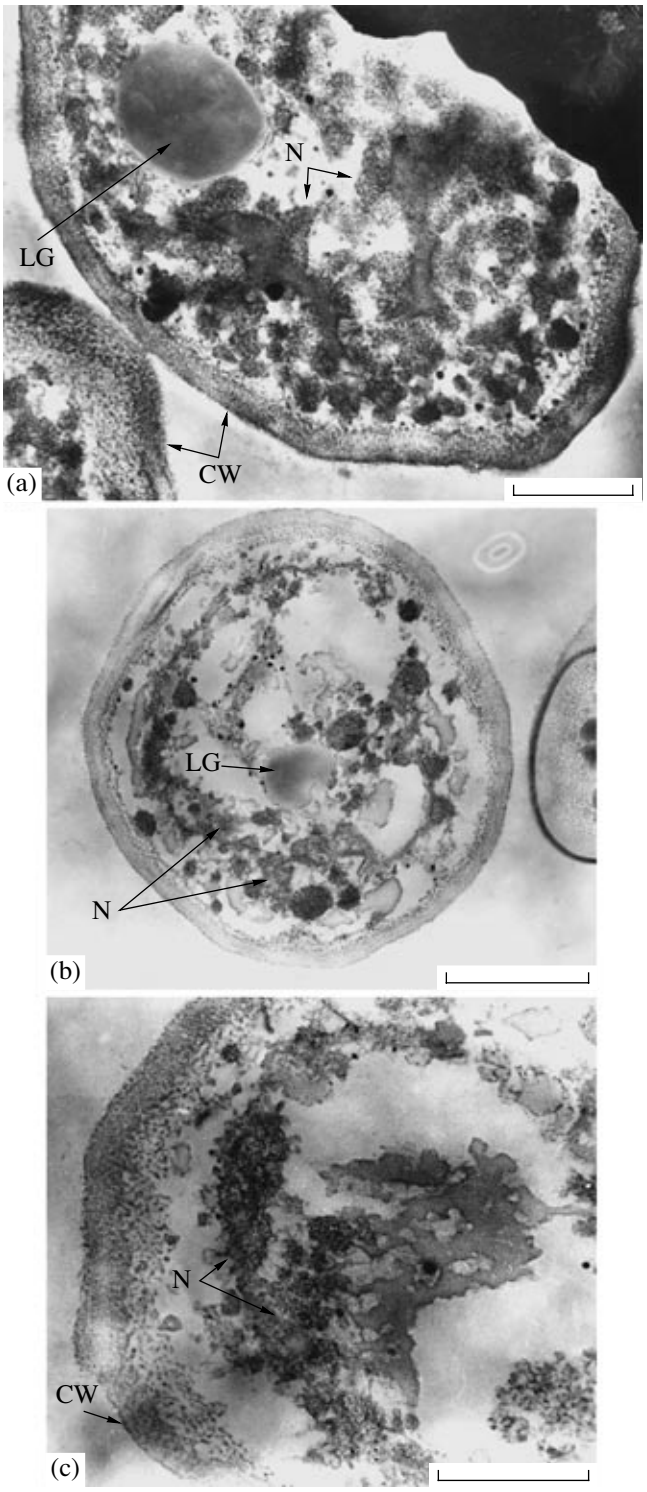


**Fig. 4.** Ultrathin sections of *S. cerevisiae* cells: (a) treated in buffer D at 37°C for 15 h and then with proteinase K; (b) (c) treated in buffer D at 100°C for 7 min and then with proteinase K. The scale bar in Fig. 4a, b is 0.5 μm, in Fig. 4c, 1 μm. Symbols are as in Fig. 3.

**Fine structure of the cells treated with chaotropic salts and proteinase K.** The study of ultrathin sections has shown (Fig. 3–7) that the application of proteinase K (PK) results in a drastic decrease in the content of electron-dense matter (stained by electron dyes) in the MM as compared to those sections treated only with CS [2]. This correlates with the disappearance of the endoplasm of the yeast cells, which is normally present in

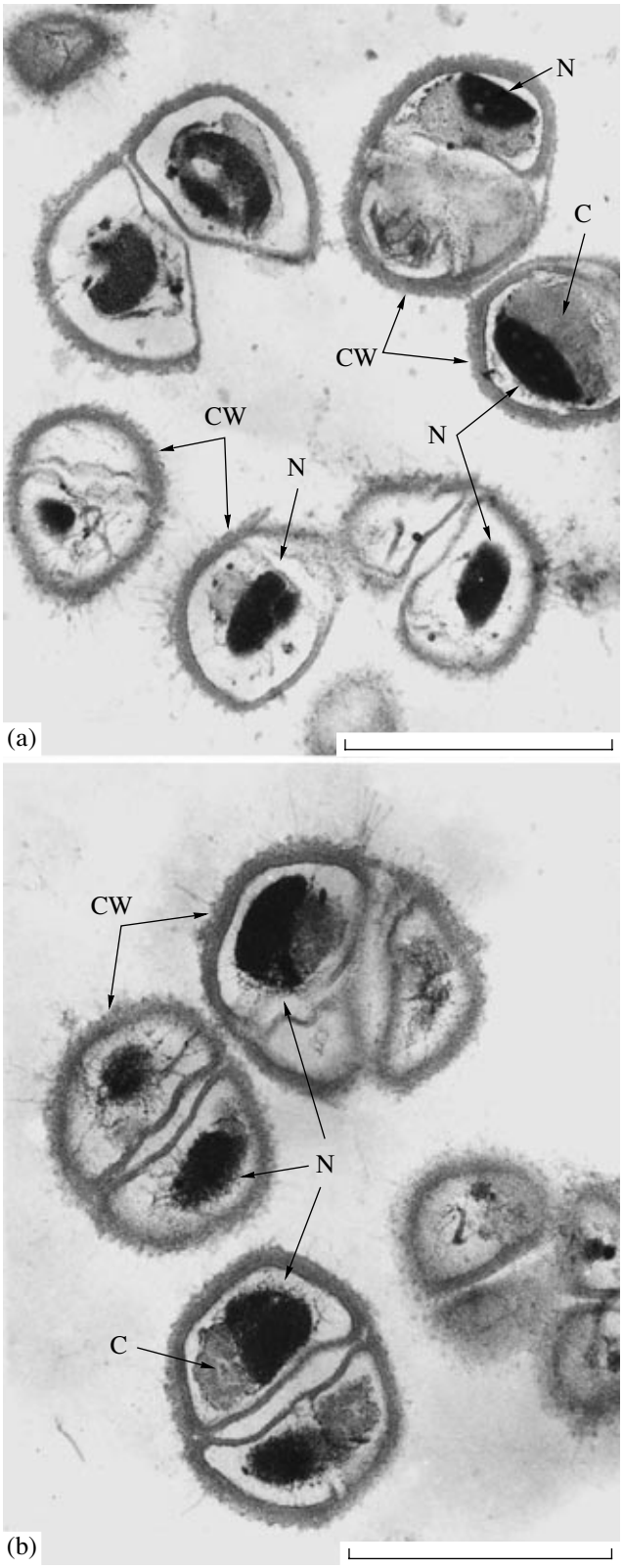


**Fig. 5.** Ultrathin sections of *M. luteus* cells: (a) treated with chaotropic salts at 37°C for 15 h and with proteinase K; (b) treated with chaotropic salts at 100°C for 10 min and with proteinase K. The scale bar is 1 μm. C, cytoplasm. Other symbols are as in Fig. 3.

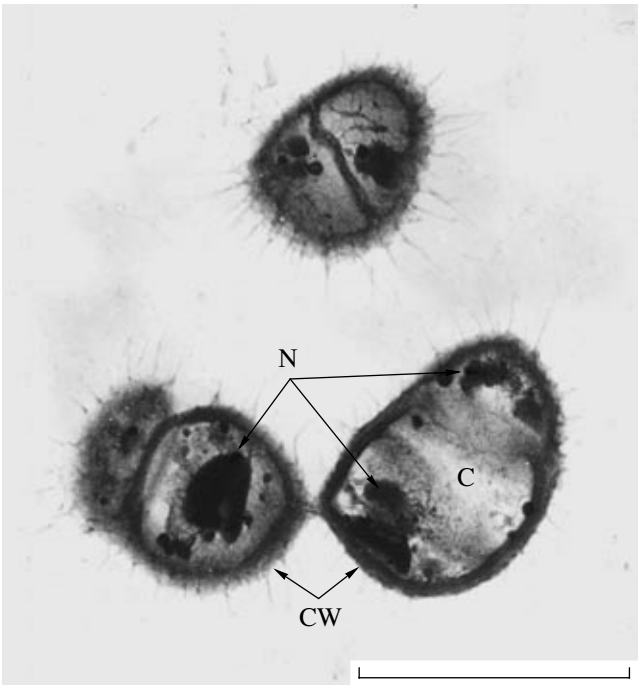


**Fig. 6.** Ultrathin sections of *A. globiformis* cells treated with chaotropic salts at 37°C for 15 h and with proteinase K. The scale bar is 1 μm. Symbols are as in Fig. 3.

yeast cells as a central electron-dense body (Fig. 3, 4) [2]. The residual cytoplasm (C) that adjoins sharply contrasting large granules of nucleic substance stained by uranyl acetate almost completely disappears or drastically decreases in bacterial MM (Fig. 5–7). These granules in the MM of *M. luteus* and *A. globiformis* obtained at 37°C have a distinctly delineated contour (Fig. 5a, 6), while in the MM treated at 100°C the surface of the granules has a fibrillar structure (Fig. 5b). The cytoplasmic remnants particularly resistant to the effect of CS and PK are visible in the yeast MM as separate fine clumps and bundles (Fig. 3, 4); however, some of the lipid granules (L) remain, having sustained CS treatment both at 37°C and at 100°C. The nucleic substance in the MM of *P. pastoris* (Fig. 3a) appears as several large bodies (probably transformed chromosomes), with a characteristic fibrillar reticular ultrastructure. The fact that DNA is a component of these bodies is demonstrated by the pronounced staining with uranyl acetate (on sections) and by DAPI staining (blue fluorescence in UV rays, fluorescence microscopy).

In the MM of *P. pastoris* obtained at 100°C (Fig. 3c), DNA most often appears as a loose net consisting of thin fibrils. In the MM of *S. cerevisiae*, the nucleic substance is dispersed as small (approximately 20–30 nm in diameter) and numerous electron-dense regions with a fibrillar fine structure, which are connected with small regions of residual cytoplasm (Fig. 4a, 4b). Another aspect of the influence of proteinase K on MM is its effect on yeast CW: its structure

loosens (Fig. 3, 4), in some cells the CW inner layer substance degrades into granules (Fig. 4c), and sometimes the thickness of CW decreases (Fig. 3c). Apparently, the resorption of CW material occurs after the first stage of its loosening.

In gram-positive bacteria *M. luterus* and *A. globiformis*, CW fine structure did not differ noticeably under CS + PK treatment (Fig. 5, 6) from that of the MM of these bacteria obtained by CS treatment alone [2]. However, PK has a very drastic effect on CW in the MM of *B. subtilis*: in a number of MM they are perforated, contain large pores (P), or even degrade into separate short fragments (leaflets) (Fig. 7). It seems that the structural proteins in *B. subtilis* play an important role in supporting the integrity of the CW murein layer. The formation of large pores upon the treatment of *B. subtilis* micromummies with proteinase K well explains the fact of DNA "leakage" revealed in the course of electrophoretic analysis of NA in combined supernatants (see Fig. 1).

**Determination of the sensitivity of PCR with CE obtained by treatment with CS and PK.** We have also determined the sensitivity of PCR with DNA-containing cell envelopes (see Material and Methods). The experiment was performed with CE of two strains of gram-positive bacteria (*A. globiformis* and *M. luteus*) with native and denatured DNA and CE of two yeast species, *S. cerevisiae* and *P. pastoris*, also with native and denatured DNA. The initial titer of yeast cells before the treatment with chaotropic salts was $5 \times 10^8$–$1 \times 10^9$; the titer of bacterial cells was much higher: $1 \times 10^{11}$.

The DNA fragments (PCR products) obtained from the yeast CE with both native and denatured DNA were revealed in all dilutions except for $10^{-7}$. They strictly corresponded in size to the expected fragments, i.e., 900 and 500 bp (not illustrated). For bacterial CE, PCR products (1500 bp) were obtained up to the $10^{-8}$ dilution. In this case, the CE with native and denatured DNA were indistinguishable in the aspect of the template properties.

The findings lead to the conclusion that each cell envelope with DNA can act as a PCR template. In other words, PCR sensitivity for the DNA-containing cell envelopes obtained using chaotropic salts and proteinase K was practically the same as in case of PCR with purified DNA.

**Optimization of conditions of the treatment of cells with chaotropic salts and proteinase K.** As follows from the results presented above, the mode of CS treatment (duration and temperature) and the time of incubation with proteinase K required optimization in some particular cases. For example, the boiling of yeast cells with CS for 5–7 min was optimal, while further treatment with proteinase K for 2.5 h resulted in intense loosening of the cell wall (at least in the case of *P. pastoris*). Thus, in this case it would be advisable to reduce the time of incubation with proteinase. This is also

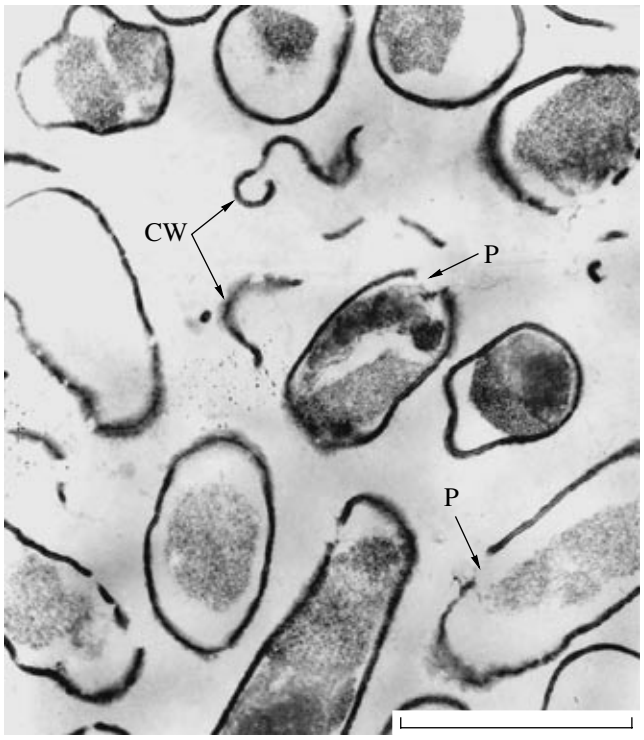


**Fig. 7.** Ultrathin sections of *B. subtilis* cells treated with chaotropic salts at 37°C for 15 h and with proteinase K. The scale is 1 μm. CW, cell wall fragments; P, cell wall pores.

applicable for the cells of gram-positive bacteria, in particular, *B. subtilis*. The cell wall of this bacterial species proved to be very sensitive to the effect of proteinase K. The treatment of MM of this microorganism with the proteolytic enzyme for 2.5 h proved to be excessive and resulted in the formation of large pores (gaps) in the cell wall. In a separate experiment we have shown that prolonged (25-h) action of proteinase K in a concentration of 100 μg/ μl resulted in the nearly complete dissolution of bacilli cell walls. It seems that cell wall proteins and peptide bridges of the murein net in bacilli are the substrates sensitive to proteinase K. Consequently, the time of treatment of *B. subtilis* MM with this enzyme should also be reduced to obtain cell envelopes with native and denatured DNA.

In compliance with the above, we have carried out experiments on optimization of the modes of treatment of bacterial and yeast cells with CS and PK. For this purpose, three tubes with equal quantities of cell biomass (approx. 30 μl) of each bacterial strain were suspended in buffer D-2 and boiled in a water bath for 5 min. The cells were precipitated by centrifugation and washed with water according to standard procedure. Washed cells (micromummies) were treated with proteinase K (50 μg/ml) in an appropriate buffer for different periods of time: 0.5, 1, and 1.5 h. On completion of the incubation, the cells were immediately washed (so that proteinase was removed) according to the standard scheme. At the final stage, the washed cell

envelopes were suspended in 300 µl of 50% ethanol. Thus, the samples of DNA-containing CE of each of the three strains of gram-positive bacteria were obtained and exposed to deep (1.5 h) and less deep (1 h and 0.5 h) action of proteinase K. The PCR sensitivity with the obtained DNA-containing cell envelopes was then determined as described in Materials and Methods. The reaction of amplification was performed in the presence of primers 27f + 1522r. The results of this experiment showed that 5-min boiling in buffer D-2 followed by 30-min incubation with proteinase K was sufficient to obtain DNA-containing CE of all three bacterial strains which could efficiently release template DNA during PCR; the PCR products were obtained from up to the $10^{-8}$ dilution (data not shown). It should be noted that the cell walls of *B. subtilis* were not degraded in such a mode.

The time of incubation with PK was optimized in a similar way for CE of the three strains of gram-positive bacteria and two yeast strains (*S. cerevisiae* and *P. pastoris*) treated with CS at 37°C. In this case, the 5-h treatment of cells in buffer D-2 at 37°C with twice-repeated changing of the buffer (after 1 and 3 h) followed by 1.5-h incubation with proteinase K was quite sufficient to obtain bacterial and yeast CE with native and deproteinized DNA. The obtained CE had a high ability to release DNA during the thermal cycles of PCR. DNA fragments (PCR products) in the course of experiments with the corresponding CE were obtained from up to the $10^{-7}$ dilution (data not shown).

Summing up this part of the work, it should be mentioned that, as a whole, the questions associated with optimization of the modes of cell treatment with CS and proteinase K can be solved quite rapidly and present no particular difficulties.

Thus, it is beyond question that the method for obtaining samples of template DNA for PCR developed in this work can be used for PCR diagnostics of infectious diseases and in taxonomic studies.

In contrast to traditional methods, the new approach is simple and makes it possible to treat tens of samples simultaneously. The whole procedure of the preparation of ten samples for PCR takes about two hours. One more advantage of the method is that CE suspensions in 50% ethanol can be stored for a long time either at –20°C or at 4°C, or even at room temperature. In addition, the results of our studies may be used to improve the method of DNA extraction by chaotropic salts from complex natural substrates (soils, silts, etc.). Thus, we have shown that DNA is retained in cell mummies of yeasts and gram-positive bacteria after the treatment with chaotropic salts (guanidinium thiocyanate, guanidinium hydrochloride). These data explain why the use of guanidinium thiocyanate results in a lower release of DNA extracted from soil as compared with other extraction agents [11]. Apparently, in this case the majority of DNA remains bound with cells and is not extracted from soils.

At present, we are studying the availability of obtained CE with native DNA for the analysis of bacterial and yeast genomes using the technique of pulse electrophoresis.

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