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EXPERIMENTAL ARTICLES

Effective PCR Detection of Vegetative and Dormant Bacterial Cells due to a Unified Method for Preparation of Template DNA Encased within Cell Envelopes

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Abstract—The unified method of template preparation for PCR in the form of DNA covered by permeabilized cell envelopes was used for the cells of different physiological status (vegetative, dormant forms of different types, and nonviable micromummies). The procedure for the preparation of template DNA included one-stage (boiling in a buffer with chaotropic salts) or two-stage (boiling in a buffer with chaotropic salts followed by treatment with proteinase K) sample preparation. The proposed method proved effective for detection of not only vegetative cells but also of the bacillary spores and the cystlike dormant cells (CLC) of nonspore-forming bacteria. For example, the two-stage sample preparation of *Bacillus cereus* spores resulted in the PCR sensitivity increase up to the detection level of 3–30 spores per sample; the one-stage sample preparation was three orders of magnitude less efficient (10^4 spores per sample). An increase in the sensitivity of PCR detection (4–10-fold) owing to the use of the two-stage sample preparation was shown for bacillary, staphylococcal, and mycobacterial CLC. The possibility of PCR detection of staphylococcal micromummies with irreversibly lost viability, which were therefore undetectable by plating techniques, was also demonstrated. The application of the unified sample preparation method ensuring efficacious PCR detection of bacterial cells, irrespective of their physiological state, may be a promising approach to more complete detection of microbial diversity and the overall insemination of natural substrates.

Keywords: dormant bacterial forms, cystlike cells (CLC), spores, DNA-containing envelopes, PCR detection sensitivity

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The efficiency of molecular-biological methods, which are widely used for assessing microbial diversity in various environmental samples and for monitoring the cell abundance, is determined by the completeness (both qualitative and quantitative) of detection of microorganisms [1]. However, when environmental objects or biological material (soil, water, clinical samples, etc.) or isolated microbial fractions are analyzed, occurrence of the cells in different physiological states, both vegetative and dormant ones, should a priori be implied. It should be noted that the possibility and sensitivity of PCR detection of different forms of dormant microbial cells remain, to a great extent, an unsolved issue. The studies in this direction were mainly conducted on the endospores of certain species of bacilli, whereas the diversity of the morphotypes of bacterial dormant forms is by far greater. For example, cystlike dormant cells (CLC) capable of long-term survival under conditions, unfavorable for growth, have been described for a number of gram-positive and gram-negative bacteria [2–6]. It is important to emphasize the prevalence of CLC in situ in environmental objects, including ancient subsoil permafrost, which was revealed by direct electron microscopic studies of the microbial populations isolated without cultivation [7, 8]. Thus, not only the vegetative cells, which may be not present in the samples (especially in the ancient ones), but also the dormant forms (DF) are of interest for the monitoring investigations and determination of microbial diversity by molecular biological methods.

Since natural microbial communities are heterogeneous by the morphological types and physiological state of constituent cells, a unified method for the preparation of DNA templates from this material is desirable. The methods successfully used for DNA isolation from the vegetative cells are not efficacious enough for the dormant forms, while the methods proposed for obtaining DNA preparations from the spores are redundant for vegetative cells. No unified method exists even for obtaining template DNA from the cells of gram-positive and gram-negative bacteria of the

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same physiological state (e.g., dividing cells). For example, while DNA extraction from the vegetative cells of gram-negative bacteria (when lysing buffers with sodium dodecyl sulfate are used) is not difficult, additional procedures of destruction are required for a number of gram-positive bacteria with rigid cell walls: mechanical disintegration, freeze–thawing, or treatment with lytic enzymes [9, 10]. Danilevich and Grishin developed a simpler and more efficient method for the preparation of template DNA samples from yeasts, fungi, and gram-positive bacteria with rigid cell walls by boiling the biomass with chaotropic salts. The preparation of DNA-containing sheaths with permeabilized cell envelopes, which can be directly used in PCR (instead of nucleic acid extraction from the cells) is a specific feature of this method [11].

The presence of thickened and often multilayered cell envelopes in the dormant forms hampers DNA isolation from them. In order to obtain the template DNA from bacillary endospores, various simple or multistage methods were used: heating the spore suspensions in water at 95–100°C [12]; incubation at 65–70°C in lysing buffers with 8 M urea, 50 mM dithiothreitol, and 1% sodium dodecyl sulfate [13] or in buffers of a different composition [14]; lysing solutions with other detergents and an additional stage of treatment with lytic enzymes [13, 15]; the combination of chemical treatment with freeze–thawing and/or mechanical disintegration of the cells [16]. As an alternative, it was also proposed to isolate DNA from the germinated cells, which have lost the dormant properties and structures [17]. The sensitivity of spore detection by PCR in the works cited above differed by several orders of magnitude (from 10^2 to 10^4 spores per sample), depending on the sample preparation method. The high level of PCR detection of the airborne *Bacillus anthracis* spores (1–10 CFU in the PCR mixture) in the sample preparation variant with simple boiling their aqueous suspension [12] contrasts sharply with the values of spore detection effectiveness after treatment according to the above-mentioned techniques.

Thus, comparison between the data on the efficiency of detection of even one type of dormant forms, the bacillary spores, is hampered by the use of different approaches to sample preparation. These findings indicate the necessity of the use of unified method for template DNA preparation, which would be universal for different types of cells. It should also be noted that the minimal spore detection threshold values in PCR in the above-cited works were calculated from the CFU titer values in the samples before sample preparation, which do not normally reflect the real number of these dormant forms. Therefore, for more complete enumeration of all the cells used for PCR analysis, it was of interest to obtain DNA-containing envelopes as both PCR templates and as objects convenient for microscopic enumeration [18].

The goal of the present work was to prepare template DNA encased in cellular envelopes from vegetative cells and dormant forms of bacteria (CLC and endospores) and to determine the efficiency of their use for PCR detection of microorganisms.

MATERIALS AND METHODS

The subjects of this study were cells of gram-positive and gram-negative spore-forming and non-spore-forming bacteria: *Bacillus cereus, Staphylococcus aureus, Mycobacterium smegmatis, Micrococcus luteus, Arthrobacter globiformis, Rhodococcus rhodochrous*, and *Azospirillum brasilense*. The cells of different physiological states were studied: vegetative, dormant, and mummified (irreversibly inactivated) (Table 1). The CLC were obtained as described earlier [2–5] by (1) long-term incubation (up to four months) of post-stationary cultures grown on N- or P-depleted media or (2) by transfer of the early stationary-phase cells into saline with 100 mM $CaCl_2$. The CLC- and spore-containing suspensions were subjected to heat treatment under the conditions lethal for the vegetative cells (Table 1). Cell viability and heat resistance were determined by the CFU number on solid media inoculated with cell suspensions; the total cell number was determined by direct microscopic count according to the techniques [2–5]. Mummified cells of *Staphylococcus aureus*, which were irreversibly inactivated but retained their external morphological integrity, were obtained by addition of the chemical analogue of the anabiosis autoinducer in the concentrations exceeding the threshold value, as described earlier [19].

Preparation of DNA encased in cell envelopes for PCR. DNA-containing cell envelopes were prepared using the one- and two-stage treatment techniques. The bacterial cells (30–50 mg dry mass) were resuspended in 400–500 μL of buffer D [11] containing 4 M of guanidine thiocyanate (Fluka, Switzerland), 25 mM of sodium citrate (pH 7.0), 0.1 M of β-mercapto-ethanol, and 0.5% N-lauroylsarcosyl sodium salt (Sigma) [11]. The suspensions were heated on a boiling water bath (5–15 min); the cells were precipitated by centrifugation (4000 *g*, 2 min); the supernatant was sampled; the pellet was washed three times with deionized water and used for PCR (method 1). For two-stage treatment (method 2), the pellet after one-stage treatment was resuspended in the buffer containing 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% N-lauroylsarcosyl sodium salt. Proteinase K (100 μg/mL, Boeringer Mannheim, Germany) was added to the suspension obtained; the mixture was incubated for 2 h at 37°C. Then the cells were precipitated by centrifugation and the sediment was washed thrice with deionized water. The pellets of cell envelopes obtained by both methods were resuspended in 200 μL of 50% ethanol and stored at 4°C.

In order to assess the DNA release in the course of one- and two-stage treatment of the cells, two volumes

Table 1. Study subjects as the sources of template DNA for PCR

Strain	Type of cells, age	Conditions of formation*	Number of cells in 1 mL of suspensions**		F, N_1/N	Number of cells in 1 mL in suspensions after heat treatment (10 min)***		F_1, N_2/N_1
			Total (N)	CFU (N_1)		*T*, ×C	CFU (N_2)	
Arthrobacter globiformis B-1112 (VKM)	VC, 24 h	LB medium	$(2.0 \pm 0.3) \times 10^8$	$(1.5 \pm 0.3) \times 10^8$	0.75	55	20	10^{-7}
	CLC, 2.5 months	C and N limit [5]	$(4.2 \pm 0.5) \times 10^8$	$(1.2 \pm 0.3) \times 10^7$	0.03	55	$(4.4 \pm 0.6) \times 10^6$	0.37
Bacillus cereus B-504 (VKM)	VC, 4 h	LB medium	$(3.1 \pm 0.4) \times 10^8$	$(2.7 \pm 0.4) \times 10^8$	0.87	60	15	5×10^{-8}
	CLC, 7 months	Medium with 6% glucose [2]	$(5.0 \pm 0.6) \times 10^8$	$(1.2 \pm 0.4) \times 10^8$	0.24	60	$(3.6 \pm 0.6) \times 10^7$	0.3
	Spores, 7 days	Medium with 0.2% glucose [2]	$(5.1 \pm 0.6) \times 10^7$	$(2.1 \pm 0.3) \times 10^7$	0.41	90	$(1.9 \pm 0.4) \times 10^7$	0.90
Micrococcus luteus NCIMB 13267	VC, 1 days	LB medium	$(2.1 \pm 0.4) \times 10^8$	$(1.6 \pm 0.3) \times 10^8$	0.76	60	<10	10^{-7}
	CLC, 2.5 months	N-depleted medium [2]	$(1.1 \pm 0.2) \times 10^8$	$(3.2 \pm 0.5) \times 10^7$	0.29	60	$(7.4 \pm 0.8) \times 10^6$	0.23
Mycobacterium smegmatis MC2 155 (ATCC 700084)	VC, 1.5 h	Sauton's medium	$(2.2 \pm 0.4) \times 10^8$	$(1.5 \pm 0.2) \times 10^8$	0.68	70	$(1.1 \pm 0.2) \times 10^3$	5×10^{-6}
	CLC, 2.5 months	N-depleted CP1 medium [4]	$(2.1 \pm 0.3) \times 10^8$	$(3.0 \pm 0.4) \times 10^6$	0.014	70	$(3.1 \pm 0.5) \times 10^4$	0.01
Staphylococcus aureus 209-P	VC, 18 h	LB medium	$(3.6 \pm 0.4) \times 10^8$	$(2.9 \pm 0.5) \times 10^8$	0.81	6060	20	5.6×10^{-7}
	CLC, 4 months	Starvation in 0.1 M $CaCl_2$ solution	$(9.0 \pm 0.9) \times 10^8$	$(1.1 \pm 0.1) \times 10^8$	0.12		$(4.2 \pm 0.5) \times 10^7$	0.38
	MM, 2 months	Introduction of C_{12}-AHB (2×10^3 M) [19]	$(5.6 \pm 0.6) \times 10^8$	0	0	–	–	–
Rhodococcus rhodochrous K-3285	VC, 1 days	LB medium	$(7.2 \pm 0.6) \times 10^7$	$(6.5 \pm 0.6) \times 10^7$	0.90	60	$(3.2 \pm 0.5) \times 10^3$	4.4×10^{-5}
	CLC, 2.5 months	N-depleted CP1 medium [4]	$(9.1 \pm 0.8) \times 10^8$	$(2.6 \pm 0.4) \times 10^8$	0.29	60	$(2.7 \pm 0.5) \times 10^7$	0.1
Azospirillum brasilense Sp7 (from the collection of the IBPPM, RAS)	VC, 2 days	Synthetic medium [3]	$(2.4 \pm 0.4) \times 10^8$	$(1.7 \pm 0.3) \times 10^8$	0.71	50	$(1.1 \pm 0.2) \times 10^2$	4.5×10^{-7}
	CLC, 4 months	N-depleted medium [3]	$(7.1 \pm 0.7) \times 10^8$	$(2.1 \pm 0.3) \times 10^8$	0.30	50	$(3.1 \pm 0.4) \times 10^7$	0.15

Notes: * The methods for obtaining CLC are described in the relevant works.

** In the case of a low total number of cells, the suspensions were concentrated 5–50-fold until to obtain the DNA templates. The N value was determined by counting the cells in a Goryaev chamber.

*** Long-stored post-stationary cultures containing the dormant forms were subjected to heat treatment to eliminate the vegetative forms; F_1 is the proportion of heat-resistant cells. Designations: VC, vegetative cells of actively growing cultures in the mid-exponential phase; CLC, cystlike dormant cells; MM, micromummies.

of 96% ethanol were added to the supernatant in order to precipitate DNA. The sediment was centrifuged (4500 *g*, 5 min), air-dried, and dissolved in 50 μL of deionized water. The presence of DNA was assessed upon electrophoretic separation (10 μL of aliquots) in 0.8% agarose gel.

PCR procedure was performed using an RTS-200 thermocycler (MJ Research, United States). The reaction mixture (50 μL) contained PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 1.5–2.0 mM $MgCl_2$), the dNTP mixture (0.2 mM each nucleotide), the primer oligonucleotides (10 pmol of each), 1 μL of DNA template suspension, and 2.5 U of thermostable *Taq*-polymerase (provided by L.I. Patrushev, Institute of Bioorganic Chemistry, Russian Academy of Sciences). The samples without template DNA were used as negative controls.

The universal primers 27f-(5' agagtttgatcctggctcag) and 1522r-(5' aaggaggtgatccarccgca) [20] were used for PCR amplification of the eubacterial 16S rRNA gene fragment. The gene-specific primers were also used for (1) the fragment of of *B. cereus* cereolysin gene *clsA* [21, 22]: Bcls-f-(5' ttccgtatgcaaagcaggcaaagg) and Bcls-r-(5' ccacgatgtaacagtccattgtgg); (2) the fragment of *S. aureus* chromosome replication initiation factor *dnaA* [23, 24]: Sdna-f-(5' aagcaccagccaaagcgtacaatcc) and Sdna-r-(5' tgaatgacggtcgtatgatcacgcc); (3) the fragment of the *M. smegmatis* sigma factor *sigF* gene (the polymerase RNA subunit) [25]: Msgf-f-(5' tacgcagacgttctcgacatgttcc) and Msgf-r-(5' atctgggtctgcgtcatcgattcg). The gene-specific primers were designed based on the nucleotide sequence of the relevant genes [21–25]; the synthesis was carried out in Evrogen and Sintol (Moscow).

The thermocycling mode was as follows: preliminary DNA denaturation, 94°C, 3 min; the cycles (27–45) of denaturation, 94°C, 30 s; annealing, 60–65°C, 30 s; synthesis, 72°C, 60–120 s; the final elongation stage: 72°C, 2 min; the primer annealing temperature was optimized in the preliminary experiments. The PCR products were analyzed electrophoretically in 0.8% agarose gel (SeaKem®, FMC Bioproducts, Sweden) using the standard Tris-acetate buffer with ethidium bromide; 1 kb DNA ladder (Fermentas, Lithuania) was used as the DNA molecular mass standard. The PCR analyses were performed in three replicates.

Microscopic observations in the epifluorescence mode were carried out using an Axioplan microscope (Carl Zeiss, Germany). In order to assess the degree of permeability of the envelopes of DNA-containing sheaths, their suspensions were stained with propidium iodide (PI) at a concentration of 3 μM and incubated in the dark at 37°C for 10 min. The proportion of red fluorescent envelopes permeable to the dye and containing DNA was determined by examining at least 20 microscope fields with at least 1000 objects for each preparation. The total number of DNA-containing envelopes in the suspensions was assessed by counting in a Goryaev chamber (at least 10 small squares). The electron microscopic studies were conducted according to the techniques described earlier [7].

RESULTS

The Preparation and Characterization of the DNA-Containing Envelopes Obtained by One- and Two-Stage Treatment of Bacterial Cells at Different Physiological States

Samples of template DNA encased in cell envelopes were obtained from the vegetative cells and the dormant forms (CLC and endospores) of bacteria using the one- and two-stage method. The specific number of DNA-containing envelopes permeable to propidium iodide (PI), a DNA-binding dye, differed in different bacteria and depended on the physiological state of cells.

Vegetative cells. With one-stage sample preparation (method 1), vegetative cells of the gram-negative bacterium *A. brasilense* were lysed even at room temperature, resulting the release of the high-polymer DNA from them, so that template preparations were obtained by precipitating it with 96% ethanol. A similar treatment supplemented with boiling of suspensions of vegetative cells of gram-positive bacteria *B. cereus, S. aureus, M. luteus, A. globiformis*, and *Rh. rhodochrous* did not result in lysis, and the cell wall was permeabilized by chaotropic salts. Cell envelopes permeable for PI that bound DNA inside were formed in each of the samples with almost 100% efficiency. However, when vegetative cells of mycobacteria *M. smegmatis* were subjected to such treatment, the content of PI-permeable envelopes was only 4–5% (Table 2).

While the two-stage treatment (method 2) of vegetative cells of gram-positive bacteria resulted in the same high yield of the DNA-containing cell envelopes as the one-stage sample preparation procedure (Table 2), the share of PI-permeable mycobacterial envelopes increased more than twofold (up to 11%). The low-molecular RNA (Fig. 1), proteins, and small quantities of DNA (less than 1% of the total amount) were present in the supernatant obtained after the precipitation of DNA-containing envelopes prepared from the vegetative cells of gram-positive bacteria (as well as *A. brasilense* CLC) (data not shown).

Dormant forms of bacteria of various species treated by method 1 or method 2 yielded the sheaths differing in the degree of permeability. When CLC of the gram-negative bacterium *A. brasilense* were treated by the one- or two-stage method, the cell envelopes were not destroyed and DNA-containing sheaths were formed. The DNA-containing cell envelopes obtained from both the CLC and endospores of *B. cereus* were permeable to PI, irrespective of the sample preparation procedure (method 1 or method 2). Similarly, cell

Table 2. Threshold of detection of bacterial cells of various physiological states in PCR with gene-specific primers using different methods for the preparation of DNA-containing cell envelopes

Cell type	Bacterial species*	Method	Number of envelopes per 1 μL of suspension	Share of the envelopes permeable to PI, %	Dilution of the envelope suspensions sufficient for PCR	Minimal detection threshold values			
						Number of envelopes per 1 μL of suspension sufficient for PCR (N_e)		CFU equivalent***	
						Total	PI-permeable	Total	PI-permeable
VC	*B. cereus*	1	$(1.3 \pm 0.3) \times 10^4$	98	10^3	13	13	11	11
		2	$(6.2 \pm 0.6) \times 10^3$	99	10^3	6	6	5.2	5.2
	S. aureus	1	$(4.0 \pm 0.5) \times 10^6$	93	10^6	4	4	3.2	3.2
		2	$(2.1 \pm 0.4) \times 10^6$	95	10^6	2	2	1.6	1.6
	*M. smegmatis***	1	$(1.7 \pm 0.4) \times 10^5$	4.5	10^2	1.7×10^3	77	1.2×10^3	54
		2	$(2.0 \pm 0.4) \times 10^5$	11	10^3	200	22	174	19
CLC	*B. cereus*	1	$(1.6 \pm 0.4) \times 10^5$	96	10^4	16	15	3.8	3.6
		2	$(1.2 \pm 0.3) \times 10^5$	97	10^5	4	4	0.8	0.8
	S. aureus	1	$(2.7 \pm 0.5) \times 10^6$	3.5	10^4	270	10	32	1.2
		2	$(2.4 \pm 0.4) \times 10^6$	30	10^5	24	7	2.9	0.8
	*M. smegmatis***	1	$(7.0 \pm 0.5) \times 10^3$	8.4	10^1	700	59	10	0.8
		2	$(1.4 \pm 0.3) \times 10^3$	6	10^1	140	8	2	0.1
Spores	*B. cereus*	1	$(2.8 \pm 0.5) \times 10^5$	97	10^1	2.8×10^4	2.7×10^4	1.1×10^4	1.1×10^4
		2	$(2.7 \pm 0.5) \times 10^5$	98	10^4–10^5	3–30	3–30	1.2–12	1.2–12
MM	*S. aureus*	1	$(1.3 \pm 0.2) \times 10^6$	95	10^5	13	12	0	0
		2	$(1.6 \pm 0.3) \times 10^6$	97	10^5	16	16	0	0

Notes: * PCR was performed with the primers specific to the *clsA* cereolysin gene fragment of *B. cereus*; the *dnaA* gene fragment of the bacterial chromosome replication initiation factor of *S. aureus*; and the *sigF* gene fragment of the sigma factor of *M. smegmatis*.

** In certain modifications of the sample preparation procedure, the threshold for detection of mycobacterial vegetative and dormant cells approached the unit values (unpublished data).

*** CFU equivalent was calculated as $N_e \times F$. The N_e values were determined by the results of counting the DNA-containing envelopes in a Goryaev chamber. The F values are shown in Table 1.

Designations: VC, vegetative cells (actively growing cultures in the mid-exponential phase); CLC, cystlike dormant cells; MM, micromummies.

envelopes with DNA were prepared from CLC of *A. globiformis, M. luteus*, and *Rh. rhodochrous*, although their degree of permeability varied from 30 to 90%, depending on the sample preparation method. For CLC of *S. aureus*, the share of permeable cell envelopes was 3.5% for the one-stage treatment and increased to 30% for the two-stage sample preparation. In the case of mycobacterial CLC (as well as for vegetative mycobacterial cells), the application of both methods provided for a rather low yield (no more than 10%) of permeable DNA-containing cell envelopes (Table 2). The moderate efficiency of permeabilization of *M. smegmatis* cells was due to high rigidity and hydrophobicity of mycobacterial cell wall [26], not the retained intactness of the cytoplasmic membrane, since the action of chaotropic agents primarily implies destruction of membrane structures [11, 18]. Due to hydrophobicity, DNA-containing envelopes of mycobacteria aggregated, which interfered with the preparation of homogeneous suspensions in serial dilutions of PCR templates.

Irreversibly inactivated cells (micromummies). The treatment of *S. aureus* micromummies using method 1 or method 2 resulted in the high (100%) yield of DNA-containing envelopes (Table 2). The loss of viability of mummified bacteria and their high permeability to PI were due to the destruction of their cytoplasmic membrane, as was shown earlier [19].

Localization of DNA inside cell envelopes shown in tests with PI was confirmed by its absence in the supernatant fluids obtained by one- or two-stage sample preparation (Fig. 1).

Thus, the efficiency of obtaining DNA-containing envelopes from vegetative cells depends on their resistance to chaotropic agents, which is determined by the specific features of the cell wall structure in different

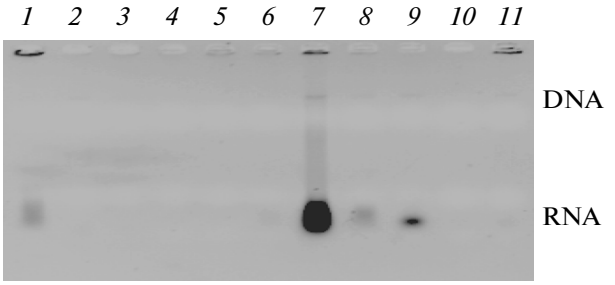


Fig. 1. Electrophoretic analysis of DNA and RNA in pooled supernatants after sedimentation and washout of DNA-containing envelopes prepared by two-stage treatment: (*1–3*) *B. cereus* vegetative cells (1 h of incubation with proteinase K) (*1*); CLC (1 h of incubation) (*2*); CLC (24 h) (*3*); (*4–6*) *M. luteus*: vegetative cells (1 h) (*4*); CLC (1 h) (*5*); CLC (24 h) (*6*); vegetative cells of *A. globiformis* (1 h) (*7*); CLC of *A. brasilense* (1 h) (*8*); (*9–11*) *Rh. rhodochrous*: vegetative cells (1 h) (*9*); stationary cells (1 h) (*10*); CLC (1 h) (*11*).

gram-positive and gram-negative bacteria. On the other hand, resistance to the sample preparation procedures depends on the morphological type of the physiologically differing cells of a given species (vegetative or dormant ones), which are substantially different in ultrastructure. This dependence was especially marked for the gram-negative bacterium *A. brasilense*: lysis of vegetative cells and release of DNA into the medium or preservation of the cell wall in the CLC and the formation of DNA-containing envelopes.

Application of DNA-Containing Envelopes for PCR

PCR with universal primers

Vegetative cells. PCR amplifications of the 16S rRNA gene fragments were successful for the initial and 10-fold diluted suspensions of DNA-containing envelopes obtained by both methods from vegetative cells of gram-positive bacteria (*M. luteus, S. aureus, Rh. rhodochrous, B.cereus*). The examples of electrophoregrams of the PCR products are shown in Figs. 2a and 2b. Note that it was necessary to dilute the initial suspensions of DNA-containing envelopes in the case of *M. smegmatis* and *A. globiformis* (Fig. 2b).

Dormant forms. With a similar PCR setup with the DNA-containing envelopes as templates prepared by both methods from CLC of all the bacteria studied, as well as from bacillary endospores, the 16S rRNA gene fragment amplicons were obtained (Figs. 2a, 2b).

Thus, the possibility of using a unified standard mode for preparation of PCR-suitable DNA templates from both vegetative cells of bacteria of different taxa with different structure and from morphologically different dormant forms was proved in principle. The possibility of detection of the dormant cystlike cells and spores in PCR with primers for the 16S rRNA gene is important for solving the tasks of microbial ecology connected with the detection of phylogenetic diversity of microorganisms in natural communities.

However, determination of the cell number in the PCR with universal primers for the 16S rRNA gene has certain limitations due to multiple copy number of this gene resulting in the obvious overestimation of the number of bacteria. For example, the number of the 16S rRNA gene copies is 12–14 for *B. cereus*; 5–6, for *S. aureus*; and 2, for *M. smegmatis* and *M. luteus*. The data on the copy number of this gene for *Rh. rhodochrous, A. globiformis*, and *A. brasilense* are absent; in some other *Arthrobacter* and *Rhodococcus* species the number of its copies varies between 4 and 6 [27]. On the other hand, determining the number of microorganisms of certain physiological groups, including those entering into the pool of dormant forms, is an important task for monitoring. For these tasks to be solved, it is preferable to use the gene-specific primers for not repeating gene sequences, rather than the universal ones.

PCR with gene-specific primers

In the experiments, PCR was performed with the primers specific to the *clsA* cereolysin gene fragment of *B. cereus*, the *dnaA* gene fragment of the bacterial chromosome replication initiation factor of *S. aureus*, and the *sigF* gene fragment of the sigma factor of *M. smegmatis*. The cell detection threshold was assessed as the highest dilutions of DNA-containing envelopes in the PCR mixture resulting in amplification of the fragments of the corresponding target genes. The cell concentration was determined by counting the DNA-containing envelopes in a Goryaev chamber.

Vegetative cells. The sensitivity of PCR with the DNA-containing envelopes obtained from *B. cereus* and *S. aureus* vegetative cells by both methods was high and corresponded to the unit number (4–16) of cells in the reaction mixture (Table 2). The inclusion of the deproteinization stage (method 2) doubled the detection sensitivity for vegetative cells of bacilli and staphylococci compared to the one-stage method 1. The sensitivity of detection of *M. smegmatis* vegetative cells treated by methods 1 and 2 was relatively low (1700 and 200 envelopes per 1 μL, respectively). Considering the efficiency of permeabilization of the envelopes in DNA-containing sheaths from vegetative cells of mycobacteria by methods 1 and 2, their detection threshold corresponded to 77 and 22 cells per 1 μL of the sample analyzed (Table 2).

Dormant forms. The two-stage treatment resulted in high detection sensitivity of *B. cereus* CLC (four DNA envelopes per 1 μL), while the one-stage one was less sensitive (16 DNA envelopes per 1 μL) (Table 2). Introduction of the deproteinization stage to increase (5–10-fold) the effectiveness of detection of *S. aureus* and *M. smegmatis* CLC (Fig. 3b, Table 2) was even more important: their detection level was

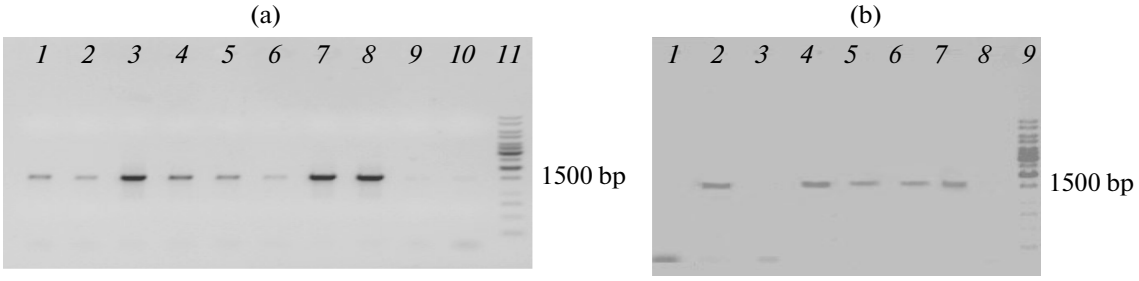
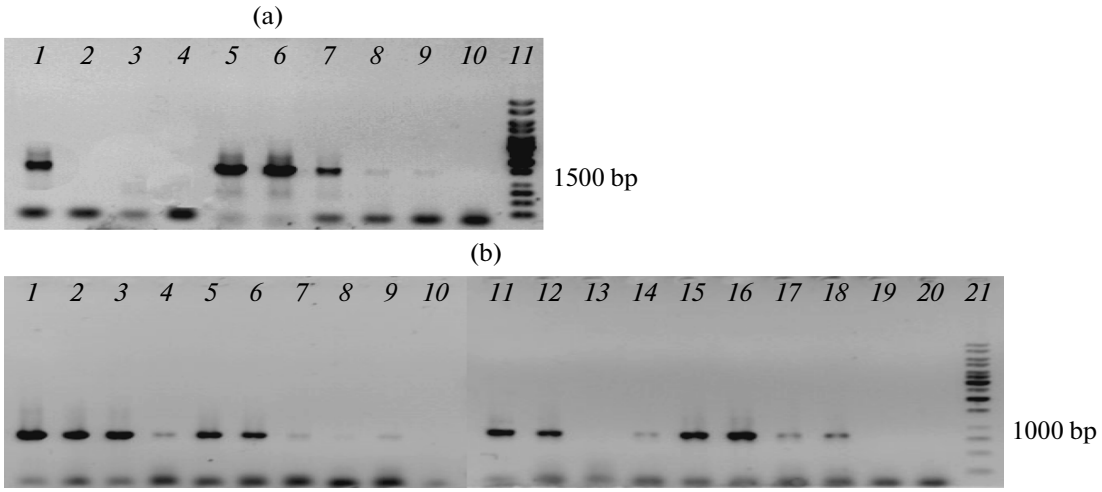


Fig. 2. Electrophoregrams of the products of PCR amplification of the 16S rRNA gene fragments with the universal primers using the DNA-containing envelopes obtained by one- and two-stage treatment of the vegetative cells and CLC: *M. luteus* (a) and *M. smegmatis* (b). Designations for (a): (*1–4*) DNA-containing envelopes from vegetative cells: one-stage (*1, 2*) and two-stage (*3, 4*) sample preparation; the initial (*1, 3*) and 10-fold diluted suspensions (*2, 4*); (*5–8*) DNA-containing envelopes from CLC (N-limited, two months): one-stage (*5, 6*) and two-stage (*7, 8*) sample preparation; the initial (*5, 7*) and 10-fold diluted suspensions (*6, 8*); negative control (without the addition of DNA templates to the PCR mixture) (*9, 10*); DNA fragment length markers (*11*). The number of cycles: 27 (*1, 3, 5, 7, 9*) and 30 (*2, 4, 6, 8, 10*). Designations for (b): DNA-containing envelopes from vegetative cells (*1–4*): one-stage (*1, 2*) and two-stage (*3, 4*) sample preparation; the initial (*1, 3*) and 10-fold diluted suspensions (*2, 4*); DNA-containing envelopes from CLC (CP1 medium, N-limited, five months) (*5–7*): one-stage (*5*) and two-stage (*6, 7*) sample preparation; the initial (*5, 6*) and 10-fold diluted suspensions (*7*); negative control (*8*); the DNA fragment length markers (*9*). The number of cycles: 27 (*1, 3, 5, 6, 9*) and 30 (*2, 4, 7, 8*).

24 DNA-containing envelopes per 1 μL when they were treated by method 2.

Efficient detection of *B. cereus* spores at a level of 3–30 DNA-containing envelopes was only achieved using the two-stage sample preparation method, whereas with the one-stage method, the detection sensitivity was less by three orders of magnitude (10^4 spores per sample) (Fig. 3a, Table 2). However, the DNA-containing envelopes obtained from spores by both methods were equally permeable to PI, which did not explain the differences in the effectiveness of their use for PCR (Table 2). To elucidate this issue, electron microscopic studies were carried out. It was shown that deproteinization determined the destruc-



Fig, 3. Electrophoregrams of the products of PCR amplification with the DNA-containing envelopes obtained using one- and two-sample preparation of (a) *B. cereus* endospores (primers for the *clsAB* gene fragment) and (b) the vegetative cells, CLC, and micromummies of *S. aureus* (primers for the *dnaA* gene fragment). Designations for (a): DNA-containing envelopes from the spores obtained with one-stage (*1–4*) and two-stage (*5–9*) sample preparation. Dilutions: (*1–4*) 10^1, 10^2, 10^3, and 10^4, respectively; (*5–9*) 10^1, 10^2, 10^3, 10^4, and 10^5, respectively; (*10*) negative control (without the addition of DNA matrices to the PCR mixture); (*11*) the DNA fragment length markers. The number of cycles: 31 (*1, 5*), 33 (*2, 6*), 36 (*3, 7*), 40 (*4, 8*) and 45 (*9, 10*). Designations for (b): (*1–4*) DNA-containing envelopes from the vegetative cells of *S. aureus* (two-stage sample preparation); dilutions: 10^3, 10^4, 10^5, and 10^6, respectively; (*5–9*) DNA-containing envelopes from CLC (two-stage sample preparation); dilutions: 10^2, 10^3, 10^4, 10^5, and 10^6, respectively; (*11–18*) DNA-containing envelopes from staphylococcal micromummies: (*11–14*) one-stage and (*15–18*) two-stage sample preparation; dilutions: 10^2 in (*11, 15*), 10^3 in (*12, 16*), 10^4 in (*13, 17*), 10^5 in (*14, 18*); (*10, 19, 20*) negative control; (*21*) the D*NA* fragment length markers. The number of cycles: 33 (*5, 11, 15*), 37 (*1, 6, 12, 16*), 41 (*2, 7, 13, 17*), and 45 (*3, 4, 8, 9, 10, 14, 18, 19, 20*).

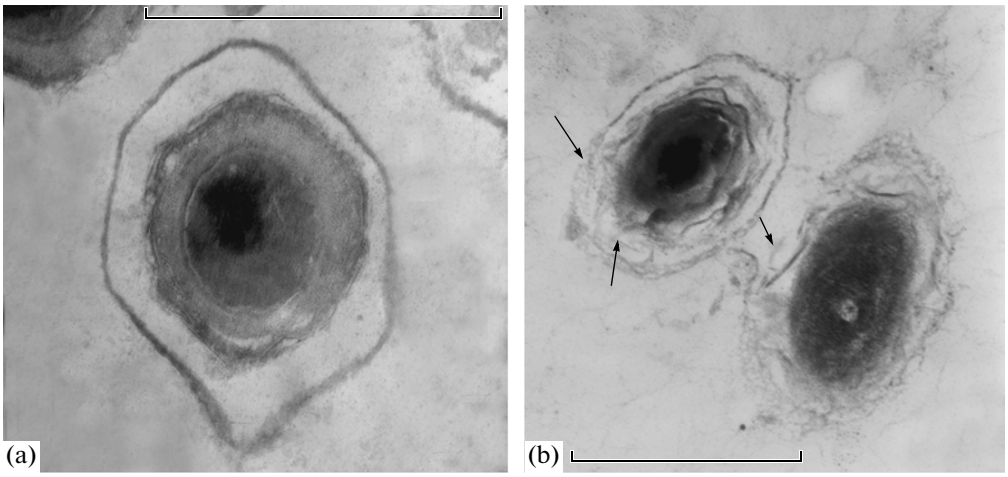


Fig. 4. Ultrathin sections of the DNA-containing envelopes prepared by one-stage (a) and two-stage (b) treatment of *B. cereus* spores. The arrows show the areas of rupture of the spore membranes. The scale bar is 1 μm.

tion of the spore coat and cortex, thus making the DNA available for PCR. The one-stage sample preparation procedure did not result in efficient disintegration of the spore coats (Fig. 4), although disorders of membrane structures caused the envelopes to be of penetrable to PI.

Irreversibly inactivated cells. The level of PCR detection of *S. aureus* micromummies with the gene-specific primers was high (13–16 DNA envelopes per 1 μL) and was comparable to the effectiveness of detection of vegetative and dormant cells of this bacterium using sample preparation method 2 (Fig. 3b, *11–18*, Table 2). Thus, the preservation of DNA suitable for PCR was revealed to occur together with the irreversible loss of viability of the mummified cells.

The values of PCR detection sensitivity reflect the total number of DNA-containing envelopes (the former cells of different physiological state) in the sample volume introduced into the reaction mixture (Table 2). Taking into account the known ratio of the CFU numbers to the total cell number in suspensions (the F value, Table 1), the detection sensitivity parameters for viable cells was also calculated as CFU equivalents ($F \times N_e$, (Table 2). For vegetative cells, the cell detection threshold values by the total number of DNA-containing envelopes and by the CFU equivalent appeared to be similar: 2 DNA envelopes and 1.6 CFU per 1 μL of the sample for *S. aureus* (method 2). However, for PCR detection of dormant CLC and endospores, the number of DNA-containing envelopes sufficient for PCR detection was 2.5–70 times higher than the corresponding CFU equivalents, e.g., 140 DNA envelopes and two CFU-equivalents for *M. smegmatis* CLC (method 2). Naturally, the CFU equivalent was zero for nonviable *S. aureus* micromummies (Table 2).

If we take into account the degree of permeabilization of DNA-containing envelopes, the values of PCR detection sensitivity for staphylococcal and mycobacterial cells appeared to be higher and approached 10^0–10^1 (Table 2). Note that the analyzed samples (the post-stationary cultures containing dormant forms, as well as a certain number of vegetative cells) were subjected to heat treatment to eliminate the latter. Considering the share of heat-resistant cells (F_1, Table 1), the sensitivity of PCR detection by the CFU equivalents was still higher: from fractions to unit values.

Thus, in assessing the detection threshold for the dormant forms and nonviable micromummies, it is more correct to take into account the total number of DNA-containing, PI-permeable envelopes rather than the CFU titer in the samples analyzed. Determination of the number of envelopes by direct count, which is implemented in this method, makes it possible to assess the level of PCR detection for the dormant forms, which is relevant to their total number in the sample.

It was noted that vegetative and dormant cells of *B. cereus* and *S. aureus* with high copy numbers of the 16S rRNA gene were detected by PCR with the universal primers when dilutions of the DNA-containing envelopes were 10 times higher than those used for PCR with the gene-specific primers. The sensitivity of detection of *M. smegmatis* cells was the same in PCR with the universal and specific primers, which was due to the low copy number of the 16S rRNA gene in the cells of mycobacteria. The detection values (by the CFU equivalents) of the vegetative cells and CLC of *A. globiformis, M. luteus, Rh. rhodochrous*, and *A. brasilense* for PCR with the universal primers were also calculated (Table 3). Since no information about the full-sized genome of these bacteria was available at the time our investigations began, it was impossible to design the gene-specific primers. Since the data on the number of the 16S rRNA gene copies are absent for certain species of these bacteria, the information

about the copy numbers of this gene in other members of the relevant genera was used for the corresponding corrections in the assessment of PCR detection for vegetative cells and CLC.

Thus, the use of the unified standard method for the preparation of template DNA in the form of DNA encased in cell envelopes ensured efficient PCR detection of bacterial cells of different physiological states.

DISCUSSION

In compliance with the goal of the study, the method for obtaining DNA templates for PCR in the form of DNA-containing cell envelopes (rather than extracted DNA) equally applicable to vegetative and dormant cells, as well as to nonviable micromummies, was successfully tried. An exception was the actively growing cells of gram-negative bacteria (exemplified by *A. brasilense*). Their low resistance to chaotropic agents used in methods 1 and 2 for obtaining DNA-containing envelopes resulted from their less rigid cell envelopes compared to those of gram-positive bacteria.

The method used is original and differs from other methods [12–16] in the absence of the stages of additional mechanical or enzymatic disintegration of the cell walls of the initial cells. The processes occurring when the cells are heated in buffer D with high chaotropic salt concentrations (one-stage sample preparation) include rapid denaturation of the cellular nucleases and other enzymatic proteins; irreversible breakdown of the membrane structures; release of lipids, some polysaccharides, proteins, and low-molecular RNA from the cells; and permeabilization of the cell envelopes. The cell envelopes formed retain the cell shape, and the DNA (99%) remains in them and is released into the PCR mixture [11, 18]. This method was used in this work for the first time for PCR detection of the dormant forms of bacteria of different taxa (Table 1).

The stage of treatment of DNA-containing envelopes with proteinase K (two-stage sample preparation) ensured an increase in the sensitivity of PCR detection 2–5-fold, 5–16-fold, and by three orders (up to 3–30 spores in the PCR mixture) for the vegetative cells, CLC, and endospores, respectively (Table 2). However, even the application of the two-stage sample preparation was insufficient to afford a high level of sensitivity for PCR detection of the vegetative and dormant cells of *M. smegmatis*, which was apparently due to the special composition and rigidity of the cell envelopes and the presence of the hydrophobic layer of mycolic acids [26], as well as significant aggregation of the cells and the DNA-containing cell envelopes prepared from them.

The threshold for PCR detection of cells in different physiological states was assessed in our study based on the results of direct enumeration of DNA-containing envelopes (the former cells), as well as on the CFU titers in the initial cell suspensions. Such combined enumeration is primarily substantiated by the discrepancies between the CFU titer and the total cell number in the samples, which is especially characteristic of long-incubated bacterial cultures (Table 1) and environmental samples [1, 4, 5, 8, 19].

Table 3. Range of PCR detection of bacterial cells of different physiological states by the CFU equivalent values (when DNA-containing cell envelopes were obtained by the two-stage method)

Species	Cell type	CFU equivalent of cell detection (per μL of analyzed suspension)
*A. globiformis**	VC	4
	CLC	15
B. cereus	VC	5.2
	CLC	4
	Spores	1.2–12
*M. luteus**	VC	2
	CLC	6
*M. smegmatis***	VC	174
	CLC	2
S. aureus	VC	1.6
	CLC	2.9
	MM	0
*Rh. rhodochrous**	VC	15
	CLC	20
*A. brasilense**	VC	1
	CLC	2

Notes: * Preliminary data were obtained for these subjects.
** When the sample preparation was improved, the threshold for detection of mycobacterial cells approached the unit values (unpublished data).

The CFU equivalents calculated for the vegetative cells, dormant forms of different types, and micromummies determined by PCR (Tables 2, 3) were used for comparison with the efficiency of detection of spores and vegetative cells by other authors. Thus, the level of detection of *B. cereus* spores (1.2–12 CFU equivalents) attained in our experiments with the use of the two-stage method was incomparably higher than the spore detection thresholds cited in [14, 17, 28] and was the same as the value shown for *B. anthracis* spores [12]. In a number of works, increased sensitivity of spore detection up to a single CFU was observed after their germination in a liquid medium [17, 28]. However, the presence of the subpopulation of deeply dormant spores not germinating under the standard conditions [29] and, consequently, the underestimation of their real number by the CFU titer

should be considered. Another example is the sensitivity of PCR detection of mycobacterial CLC in the two-stage sample preparation variant, which corresponded to unit CFU equivalent values (2 per 1 μL of the sample), although the total number of the envelopes obtained from the CLC (sufficient for PCR detection) was substantially higher (140 per 1 μL) (Table 2). Thus, the use of DNA-containing envelopes as both templates in the PCR and as subjects convenient for direct microscopic count made it possible to assess the number of dormant forms in the sample analyzed with higher accuracy, which is especially important for dormant cells poorly germinating on solid media. It should be noted that the combined estimate of the efficiency of PCR detection of the vegetative cells may be excessive in a number of cases. For example, in our experiments, the detection threshold of actively growing staphylococci corresponded to two DNA envelopes or 1.6 CFU equivalents (Tables 2, 3), which coincided with the efficiency of their detection (1.7 CFU) by quantitative PCR [30].

As for the microbial ecology issues, an important result was the comparability between the sensitivity of PCR detection of the vegetative and dormant cells (method 2) of bacteria of the same species (Tables 2, 3), which implies the possibility of determination of the total number of microorganisms in natural populations, which are heterogeneous by the physiological state of the cells. This is important for more complete detection of the species diversity and the microbial abundance in the soil, water, and clinical samples, as well as in ancient microbial communities, such as subsoil permafrost where CLC predominate [7, 8]. The possibility of efficient DNA amplification in PCR using the proposed method for the environmental samples with a low or zero number of the colony-forming cells should be noted. In our experiments, these situations were simulated by the use of suspensions of the poorly germinating CLC of a number of bacteria or of micromummies. The possibility of PCR detection of micromummies, the cell forms with irreversibly lost viability, which are, in fact, the memory of the earlier biocenosis composition, is essentially important for analysis of ancient objects. Structures practically identical to the mummified forms of microorganisms in laboratory cultures were detected in the samples of fibrous kerites [19].

Thus, the methods proposed in order to increase the efficiency of the PCR analysis of microbial populations and tested for both vegetative cells and different types of dormant forms and mummified cells seem to be promising in unraveling the hidden reserves of the present and past microbial life.

REFERENCES

1. Amman, R.I., Ludwig, W., and Schleifer, K.H., Phylogenetic identification and in situ detection of individual microbial cells without cultivation, *Microb. Rev.*, 1995, vol. 59, no. 1, pp. 143–169.
2. Mulyukin, A.L., Lusta, K.A., Gryaznova, M.N., Kozlova, A.N., Duzha, M.V., Duda, V.I., and El'-Registan, G.I., Formation of resting cells by *Bacillus cereus* and *Micrococcus luteus, Microbiology*, 1996, vol. 65, no. 6, pp. 683–689.
3. Mulyukin, A.L., Suzina, N.E., Pogorelova, A. Yu., Antonyuk, L. P., Duda, V.I., and El-Registan, G.I., Diverse morphological types of dormant cells and conditions for their formation in *Azospirillum brasilense, Microbiology*, 2009, vol. 78, no. 1, pp. 33–41.
4. Mulyukin, A.L., Kudykina, Yu.K., Shleeva, M.O., Anuchin, A.M., Suzina, N.E., Danilevich, V.N., Duda, V.I., Kaprelyants, A.S., and El-Registan G.I., Intraspecies diversity of dormant forms of *Mycobacterium smegmatis, Microbiology*, 2010, vol. 79, no. 4, pp. 461–471.
5. Demkina, E.V., Soina, V.S., El'-Registan, G.I., and Zvyagintsev, D.G., Reproductive resting forms of *Arthrobacter globiformis, Microbiology*, 2000, vol. 69, no. 3, pp. 309–313.
6. Garduño, R.A., Garduño, E., Hiltz, M., and Hoffman P.S., Intracellular growth of *Legionella pneumophila* gives rise to a differentiated form dissimilar to stationary-phase forms, *Infect. Immun.*, 2002, vol. 70, pp. 6273–6283.
7. Suzina, N.E., Mulyukin, A.L., Kozlova, A.N., Shorokhova, A.P., Dmitriev, V.V., Barinova, E.S., Mokhova, O.N., El'-Registan, G.I., and Duda, V.I., Ultrastructure of resting cells of some non-spore-forming bacteria, *Microbiology*, 2004, vol. 73, no. 4, pp. 435–447.
8. Soina V.S., Mulyukin A.L., Demkina E.V., Vorobyova E.A., El-Registan G.I. The structure of resting microbial populations in soil and subsoil permafrost, *Astrobiology*, 2004, vol. 4, no. 3, pp. 345–358.
9. Picard, C., Ponsonnet, C., Paget, E., Nesme, X., and Simonet, P., Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction, *Appl. Environ. Microbiol.*, 1992, vol. 58, no. 9, pp. 2717–2722.
10. Miller, D.N., Bryant, J.E., Madsen, E.L., and Ghiorse, W.C., Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples, *Appl. Environ. Microbiol.,* 1999, vol. 65, no. 11, pp. 4715–4724.
11. Danilevich, V.N. and Grishin E.V., A new approach to the isolation of genomic DNA from yeast and fungi: preparation of DNA-containing cell envelopes and their use in PCR, *Russ. J. Bioorg. Chem.*, vol. 28, vol. 2, pp. 136–146.
12. Makino, S. and Cheun, H.I., Application of the real-time PCR for the detection of airborne microbial pathogens in reference to the anthrax spores, *J. Microbiol. Meth.*, 2003, vol. 53, no. 2, pp. 141–147.
13. Nicholson, W.L. and Setlow, P., Dramatic Increase in Negative superhelicity of plasmid DNA in the forespore compartment of sporulating cells of *Bacillus subtilis, J. Bacteriol.*, 1990, vol. 172, no. 1, pp. 7–14.
14. D'Alessandro, B., Antúnez, K., Piccini, C., and Zunino, P., DNA Extraction and PCR detection of

Paenibacillus larvae spores from naturally contaminated honey and bees using spore-decoating and freeze-thawing techniques, *World J. Microbiol. Biotechnol.*, 2007, vol. 23, pp. 593–597.

15. Ryba, S., Titera, D., Haklova, M., and Stopka, P., A PCR Method of detecting american foulbrood (*Paenibacillus larvae*) in winter beehive wax debris, *Vet. Microbiol.*, 2009, vol. 39, no. 1–2, pp. 193–196.
16. Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K., and Jackson, P.J., Small-scale DNA sample preparation method for field pcr detection of microbial cells and spores in soil, *Appl. Environ. Microbiol.*, 1998, vol. 64, no. 7, pp. 2463–2472.
17. Cheun, H.I., Makino, S.-I., Watarai, M., Erdenebaatar, J., Kawamoto, K., and Uchid, I., Rapid and effective detection of anthrax spores in soil by PCR, *J. Appl. Microbiol.*, 2003, vol. 95, no. 4, pp. 728–733.
18. Danilevich, V.N., Duda,V.I., Suzina, N.E., and Grishin, E.V., Obtaining and characterization of DNA-containing micromummies of yeasts and gram-positive bacteria with enhanced cell wall permeability: application in PCR, *Microbiology*, 2007, vol. 76, no. 1, pp. 60–69.
19. Suzina, N.E., Mulyukin, A.L., Loiko, N.G., Kozlova, A.N., Dmitriev, V.V., Shorokhova, A.P., Gorlenko, V.M., Duda, V.I., and El'-Registan, G.I., Fine structure of mummified cells of microorganisms formed under the influence of a chemical analogue of the anabiosis autoinducer, *Microbiology*, 2001, vol. 70, no. 6, pp. 667–677.
20. Johnson, J.L., Similarity Analysis of RNAs, in *Methods for General and Molecular Bacteriology*, Gerhardt, P., Murray, R.G.E., Wood, W.A., and Krieg, N.R., Eds., Washington: Amer. Soc. Microbiol., 1994, pp. 683–700.
21. Schraft, H. and Griffiths, M.W., Specific oligonucleotide primers for detection of lecithinase-positive *Bacillus* spp. by PCR, *Appl. Environ. Microbiol.*, 1995, vol. 61, no. 6, pp. 98–102.
22. Gilmore, M.S., Cruz-Rodz, A.L., Leimeister-Wächter, M., Kreft, J., and Goebel, W., A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage, *J. Bacteriol.*, 1989, vol. 171, no. 2, pp. 744–753.
23. Katayama, H., Mizushima, T., Miki, T., and Sekimizu, K., Molecular cloning and sequence analysis of the *dnaA* gene of *Staphylococcus aureus, Biol. Pharm. Bull.*, 1997, vol. 20, no. 7, pp. 820–822.
24. Holden, M.T.G., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R., Barron, A., Bason N., Bentley, S.D., Chillingworth, C., Chillingworth, T., Churcher, C., Clark, L., Corton, C., Cronin, A., Doggett, J., Dowd, L., Feltwell, T., Hance, Z., Harris, B., Hauser, H., Holroyd, S., Jagels, K., James, K.D., Lennard, N., Line, A., Mayes, R., Moule, S., Mungall K., Ormond, D., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Sharp, S., Simmonds, M., Stevens, K., Whitehead, S., Barrel,l B.G., Spratt, B.G.,and Parkhill, J., Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, vol. 101, no. 26, pp. 9786–9791.
25. Fleischmann, R.D., Dodson, R.J., Haft, D.H., Merkel, J.S., Nelson, W.C., and Fraser, C.M., *Mycobacterium smegmatis* str. MC2 155, complete genome, *Direct Submission to GenBank: CP000480.1 on 2006. Oct.19.*
26. Brennan, P.J. and Nikaido, H., The envelope of mycobacteria, *Annu. Rev. Biochem.,* 1995, vol. 64, pp. 29–63.
27. http//ribosome.mmg.msu.edu//rrndb
28. Lara-Reyna, J., Olalda-Portugal, V., and Olmedo-Alvarez, G., An Efficient procedure for the isolation of PCR-competent DNA from *Bacillus* endospores germinated in soil, *World J. Microbiol. Biotechnol.*, 2000, vol. 16, no. 4, pp. 345–351.
29. Ghosh, S. and Setlow, P., Isolation and characterization of superdormant spores of *Bacillus* species, *J. Bacteriol.*, 2009, vol. 191, no. 6, pp. 1787–1797.
30. Trnčíková T., Hrušková V., Oravcová K., Pangallo D., Kaclíková E., Rapid and sensitive detection of *Staphylococcus aureus* in food using selective enrichment and real-time PCR targeting a new gene marker, *Food. Anal. Meth.*, 2009, vol. 2, no. 4, pp. 241–250.