ISSN 0026-2617, Microbiology, 2007, Vol. 76, No. 4, pp. 405–412. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © A.M. Shadrin, E.V. Shapyrina, A.V. Siunov, K.V. Severinov, A.S. Solonin, 2007, published in Mikrobiologiya, 2007, Vol. 76, No. 4, pp. 462–470.

EXPERIMENTAL ARTICLES

Bacillus cereus Pore-forming Toxins Hemolysin II and Cytotoxin K: Polymorphism and Distribution of Genes among Representatives of the Cereus Group

A. M. Shadrin[a], E. V. Shapyrina[a], A. V. Siunov[a], K. V. Severinov[b,c], and A. S. Solonin[a,1]

[a] *Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS, Prospect Nauki, 5, Pushchino, Moscow Region, 142290 Russia*
[b] *Institute of Molecular Genetics, Kurchatov sq., 2, Moskow 123182 Russia*
[c] *Waksman Institute of Microbiology, Rutgers, The State University of New Jercy, 190 Frilinghuysen id., Lisataway, NJ 08854, USA*

Received November 2, 2006

Abstract—Phylogenetic interrelation between 40 strains of the *Bacillus cereus* group has been established using BcREP fingerprinting. The PCR method has shown that the frequency of occurrence of the genes of cytotoxin K (*cytK*) and hemolysin II (*hlyII*) is 61% and 56%, respectively, and the gene of the hemolysin II regulator (*hlyIIR*) occurs together with *hlyII*. Comparison of the results of fingerprinting, PCR, and RFLP of the toxin genes showed that bacteria with the $hlyII^+$ and $cytK^+$ genotypes did not form separate clusters. However, microorganisms with the similar fingerprints were shown to have toxin genes of the same type. The proposed variant of RFLP analysis made it possible to clearly distinguish between the *cytK1* and *cytK2* genes. Twenty-three strains having the *cytK* genes carried no *cytK1* dangerous for mammals. Additionally, the entire collection of microorganisms was tested for the ability to grow at 4°C. This property was revealed for five strains, which should most likely be classified as *B. weihenstephanensis*. Two of the five psychrotolerant microorganisms carried the hemolysin II gene variant of the same type according to RFLP. None of the five strains had the *cytK* gene. These strains did not form close groups upon clustering by the applied method of Bc-REP fingerprints.

Key words: B. cereus, hemolysin II, cytotoxin K, hemolysin II regulator, fingerprinting.

DOI: 10.1134/S0026261707040042

The cereus group of bacilli includes *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus anthracis*, *Bacillus mycoides*, as well as *Bacillus pseudomycoides* and *Bacillus weihenstephanensis*, which have been recently described as independent species. This group is represented by soil gram-positive rod-shaped spore-forming microorganisms [1]. The anthrax pathogen *B. anthracis* produces a number of lethal toxins and forms a poly-D-glutamic capsule. *B. thuringiensis* forms parasporal crystals which make it pathogenic for insects. The distinctive feature of *B. mycoides* is the specific rhizoid form of its colonies [2]. *B. cereus* is a widespread opportunistic pathogen which often causes food poisoning [1]. All four of these species had already been described by the beginning of the 20th century. The methods of genosystematics such as DNA-DNA hybridization and 16S rRNA gene sequencing, the results of which have become the basic criteria of microbial classification, suggest considering the above four species as a single one [3]. However, the previous species names have been retained due to some social and economical reasons. Moreover, two more separate species have recently been isolated within the group: *B. pseudomycoides*, different from *B. mycoides* in fatty acid composition [4]; and *B. weihenstephanensis*, different from other representatives of the cereus group in the ability to grow at low temperatures [5].

The current data on biodiversity of the *B. cereus* group and improvement of typing methods have created the necessary prerequisites for the emergence of novel approaches to classification of the microorganisms of this group. Different variants of multilocus typing (MLST) have been used in recent years, making it possible to identify the clusters that confirm the clonal character of populations of *B. anthracis* [6, 7]; *B. cereus*, which is pathogenic for mammals [6, 7, 8]; and insecticidal representatives of this group [8]. In addition, the panmictic character of the population of soil psychrotolerant microorganisms has been revealed [8]. However, despite all the progress made, including the analysis of complete genomes of individual members of this group, it has been impossible as yet to elab-

[1] Corresponding author; e-mail: solonin@ibpm.pushchino.ru.

orate universal distinct criteria for distinguishing one species from another [1].

The phenotypic characteristics of cereus bacteria, originally underlying their classification (the complex of lethal toxins and the poly-D-glutamic capsule of *B. anthracis*, insecticidal toxins of *B. thuringiensis*), are encoded by the genes located on macroplasmids [9]. Apart from these specific toxins, members of the cereus group produce a diversity of toxins, which are encoded by genes of chromosomal location. For example, the chromosome of strain *B. cereus* ATCC 14579^T was shown to carry the genes of the following toxins: hemolysin BL, nonhemolytic enterotoxin, enterotoxin T, enterotoxin FM1, cytotoxin K, phosphatidylinositol-specific phospholipase C, phosphatidylcholine-specific phospholipase C and sphingomyelinase (or cereolysin AB), cereolysin O, hemolysin II, and hemolysin III [10]. Expression of most of these is regulated by the pleiotropic transcriptional activator PlcR by the "quorum sensing" mechanism [10, 11].

Quite recently, there has been a severe food poisoning outbreak, with fatalities. The pathogenic microorganism was strain *B. cereus* 391-98, which is able to produce only one of the known *B. cereus* enterotoxins: cytotoxin K (CytK). CytK has been shown to have necrotic and hemolytic effects and to be toxic for human intestine epithelium cells [12]. Cytotoxin K secreted by this strain (CytK1) was different in amino acid sequences and pore-forming properties from cytotoxins K of less hazardous strains (CytK2) [13].

Some microorganisms of the *B. cereus* group produce hemolysin II (HlyII) [14, 15]. The amino acid sequence of HlyII is similar to the sequences of CytK (up to 37% identity). Both toxins belong to the family of β-folded pore-forming toxins (β-PFT) [13]. Other members of this family are alpha-hemolysin, leucocidins, gamma-hemolysin of *Staphylococcus aureus*, and beta-toxin of *Clostridium perfringens* [16]. The identity of the amino acid sequences of these proteins is 20–30%. In contrast to the *cytK* gene, the expression of *hlyII* is not regulated by PlcR but controlled by transcriptional regulator HlyIIR, the gene of which is located immediately after the *hlyII* gene [17].

In this work, we describe the character of distribution of β-PFT genes *cytK* and *hlyII* and the regulatory gene *hlyIIR* among the members of the cereus group. We also demonstrate how RFLP variants of the genes of these toxins correlate with the clustering of the studied microorganisms on the basis of REP PCR analysis.

MATERIALS AND METHODS

Bacterial strains. Twenty-four *B. cereus* strains and twelve *B. thuringiensis* strains were obtained from the All-Russian Collection of Microorganisms (VKM). Strains *B. weihenstephanensis* KBAB4, *Bacillus thuringiensis* (serovar *israelensis*) ATCC 35646, and the type strain *B. cereus* 6A5^T (from Bacillus Genetic Stock Collection) were provided by A. Sorokin (Institut National de la Recherche Agronomique, France). Strain *B. cereus* ATCC 11778 was obtained from the "Difco spores" commercial preparation (Difco).

Detection of psychrotolerant strains. To reveal psychrotolerant microorganisms, strains were cultured for ten days at 4°C and 8°C on agarized LB medium and on LB containing 5% human erythrocytes.

Group-specific BcREP-PCR fingerprinting. DNA was isolated by the method described in "Current Protocols in Molecular Biology" [18]. PCR was performed according to the conditions proposed in the work [19] on a PalmCycler CG1-96 (Corbet Research). After PCR, 4-μl samples were placed into wells (5 × 0.5 mm) in a 12.5 × 8.5 cm 1.5% agarose gel. Electrophoresis was carried out at electric field strength of ~2 V/cm in 40 mM Tris-acetate buffer (TAE) for 5 h. After electrophoresis, the gel was placed for 30 min into ethidium bromide solution (0.5 μg/ml) and photographed with the Kodak EDAS 290 Gel Documentation System (Kodak). The molecular weights of polymorphic amplicons in the range of 500 to 4000 bp were determined using Totallab 2.0 software, according to molecular weight standards. The results were converted into a binary (1/0) matrix. Cluster analysis was performed using Treecon 1.3 software [20]. The similarity of the fingerprints of the strains was assessed according to [21]. UPGMA was used as a clustering algorithm.

Analysis of conservative and polymorphic regions of the *cytK* and *hlyII* gene sequences. The AlignX software (Vector NTI 9.0 application) was used for alignment of the seven known sequences of the *hlyII* genes (*B. thuringiensis* serovar *konkukian* 97-27 (AE017335.1), *B. anthracis* 'Ames Ancestor' (AE017334.2), *B. anthracis* Ames (AE016879.1), *B. cereus* ATCC 14579^T (AE016877.1), *B. cereus* E33L (CP000001.1), *B. anthracis* Sterne (AE017225.1), *B. cereus* VKM B-771 (U94743.1)), and 8 sequences of the *cytK* genes (*cytK-2* of *B. cereus* 23 (AJ318877.2), *cytK-2* of *B. cereus* 1230-88 (AJ318876.2), *cytK-2* of *B. cereus* FM-1 (AJ318875.3), *cytK* of *B. thuringiensis* serovar *konkukean* 97-27 (AE017355.1), *cytK-1* of *B. cereus* 391-98 (AJ277962.1), *cytK-2* of *B. cereus* ATCC 10987 (AE017194.0), *cytK-2* of *B. cereus* ATCC 14579^T (AE016877.1), and *cytK* of *B. cereus* E33L (CP000001.1)). Sequence numbers in GenBank are given in brackets. The polymorphic and conservative gene regions were revealed by results of alignments for each of the genes.

Detection of *cytK, hlyII*, and *hlyIIR* by PCR. Primers for PCR detection of the genes (see the table) were developed on the basis of data on conservative gene regions. The β-PFT genes were detected by four variants of PCR using different combinations of primers CF1, CF2, CR1, and CR2 for *cytK* and HF1, HF2, HR1, and HR2 for *hlyII*. The *hlyIIR* gene was detected using primers RF1, RF2, and RR1.

Primers for gene detection

Primer	Sequence	Amplified gene
RF1	ATGGGGAAGTCTCGTGAGCAGA	*hlyIIR*
RR2	AAAACAATAGAAGTGATCCAATGTATCGT	*hlyIIR*
RF1	GTAAATGTCGCTGATGCTTCATACTAT	*hlyIIR*
HF2	TGGGGGATACTATAACGCAGGTTT	*hlyII*
HR3	CGTTCCTTCATTATAACTACGTGATTTCAT	*hlyII*
HF1	ATGAAAAAAGCAAAGGGAATAGCTAAAA	*hlyII*
HR4	TTAGATTTTTTTAATCTCAATATAAGGTCCTTT	*hlyII*
CF2	CGCTATCCATCACAATATGATATTGCA	*cytK*
CR3	GTGTAAATGCCCCAGTTTTGATTATTATAT	*cytK*
CF1	GTTGTAACAGATATCGGTCAAAATGCA	*cytK*
CR4	AACCCAGTTACCAGTTCCGAATGT	*cytK*

The reaction mixture for PCR (10 μl) contained 1× buffer for Taq polymerase (Promega), 1.5 mM $MgCl_2$, 0.5 U Taq polymerase, deoxynucleotide triphosphates (0.2 mM each) (analytical grade, Bioline), oligonucleotide primers (0.2 μM each), and DNA template (4 ng). Temperature mode of reactions: 94°C, 5 min; 3 repeats: (94°C, 40 sec; 45°C, 40 sec; 72°C, t1 sec); 32 repeats: (94°C, 20 sec; 52°C, 20 sec; 72°C, t2 sec); elongation time t1 = 40 sec, t2 = 30 sec for primers RF1+RR1, HF2+HR1, CF2+CR1, and RF2+RR1; t1 = 60 sec, t2 = 45 sec for HF1+HR1 and CF1+CR1; t1 = 75 sec, t2 = 50 sec for HF2+HR2, CF1+CR2, and CF2+CR2; t1 = 90 sec, t2 = 65 sec for HF1+HR2.

PCR products were analyzed in gels with agarose concentration of 1.5 to 2.5%. The detected gene was considered to be present if a product with the molecular weight corresponding to the calculated one was detected in at least one PCR variant.

RFLP analysis of *cytK* and *hlyII*. The regions of the *cytK* and *hlyII* genes were amplified using primers CF1+CR2 and HF2+HR2, respectively. To minimize the yield of nonspecific products, PCR was performed in the optimized temperature mode: 94°C for 5 min; and 40 repeats: (94°C, 25 sec; 57°C, 25 sec; 72°C, 25 sec). The volume of the reaction mixture was 50 μl. The specificity and homogeneity of the amplification products was confirmed by electrophoresis in 1.5% agarose gel. Restriction endonucleases *Sau*3AI (laboratory collection), *Rsa*I (laboratory collection), and *Alu*I (Sibenzyme) were used for hydrolysis of amplicons. For hydrolysis, 6 μl of PCR products was added to 6 μl of the solution containing 1–5 activity units of restriction endonuclease in the double-strength reaction buffer Yellow tango+ (Sibenzyme or Fermentas). Hydrolysis was carried out for 1 h. The hydrolysis products of *cytK* and *hlyII* amplicons were separated in 15% and 6% polyacrylamide gels, respectively. Electrophoresis was performed in 1× TAE buffer at electric field strength of ~2–3 V/cm. After electrophoresis, the gel was stained in ethidium bromide solution (0.5 μg/ml).

The genes with similar positions of the *Sau*3AI, *Rsa*I, and *Alu*I sites on the amplicon were considered as a separate RFLP group. Positions of restriction sites for the *hlyII* gene in strains *B. thuringiensis* 97-27, *B. anthracis* 'Ames Ancestor', *B. anthracis* Ames, *B. cereus* ATCC 14579^T, *B. cereus* E33L, *B. anthracis* Sterne, *B. cereus* VKM B-771, and *cytK* of strains *B. cereus* 23, *B. cereus* 1230–88, *B. cereus* FM-1, *B. thuringiensis* 97-27, *B. cereus* 391-98, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579^T, and *B. cereus* E33L were determined on the basis of the nucleotide sequences available in GenBank.

Treecon 1.3 software was used to construct the dendrograms. The operational taxonomic unit (OTU) was the presence/absence of a particular site of restriction endonucleases (*Alu*I, *Sau*3AI, and *Rsa*I) in the particular position of an amplicon. The similarity was calculated by simple matching. Genes were grouped by the neighbor-joining method [22].

RESULTS AND DISCUSSION

Group-specific Bc-REP fingerprinting. The collection of strains described in this paper includes a part of VKM strains and contains members of the *B. cereus* group isolated from natural sources over the course of the 20th century. Historically, isolates used to be classified by species affiliation mainly on the basis of their phenotypic characters. Thus, the formation of parasporal crystals was an indication to identify a strain as *B. thuringiensis*; in the case of their absence, a microorganism was classified as *B. cereus*. The species affiliation of most strains described in this paper was deter-

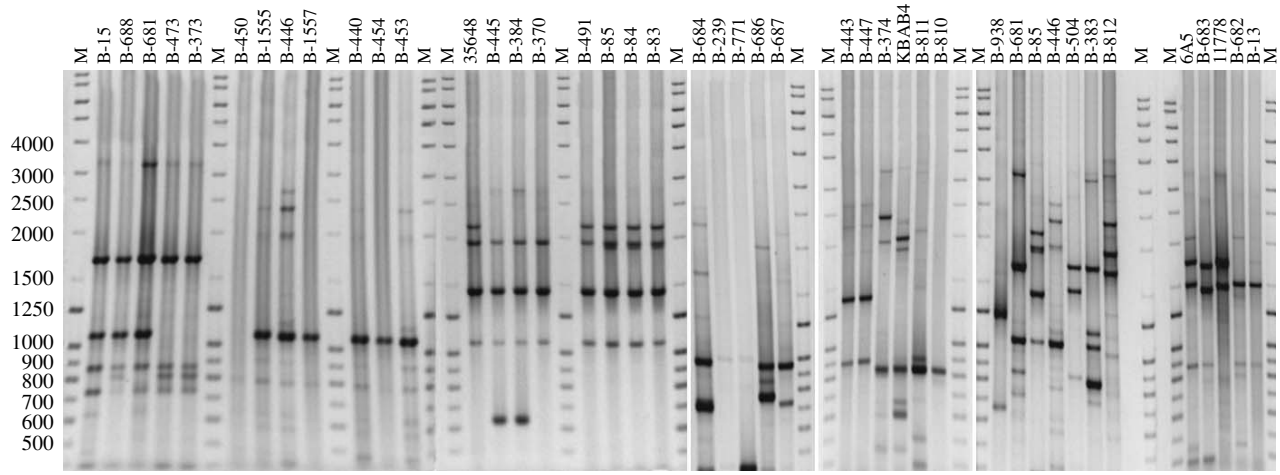

Fig. 1. Electrophoregram of BcREP-PCR fingerprints. Collection numbers of the cereus group strains are given above the tracks. M, markers of DNA molecular mass (the length of marker fragments in base pairs is indicated on the left of the figure). Collection strain numbers are given without the acronyms specifying the collection.

mined on the basis of phenotypic criteria. However, such classification of isolates has been disputed in many works and conflicts with the modern view of determination of the relationship between the members of the *B. cereus* group [5, 7, 9]. We have therefore decided to use a variant of typing based on the group-specific Bc-REP fingerprints intended particularly for the microorganisms of the *B. cereus* group [19]. This method was shown to have a high resolution and to distinguish between *B. thuringiensis* serotypes.

The analysis of Bc-REP PCR fingerprints on an electrophoregram showed 4 to 17 amplified DNA fragments of 500 to 4000 bp (Fig. 1). The similarity between 40 fingerprints was 10 to 100%. We determined four groups of bacterial isolates with the similarity of 70 to 100% within the groups. Each group contained 4–8 strains (Fig. 2). Comparison of the species affiliations of the strains with the fingerprinting results revealed that clusters "A", "C", and "D" comprised microorganisms of only one species, and cluster "B" comprised four strains of *B. cereus* and four strains of *B. thuringiensis*. Note that cluster "D" includes two subcultures of the type strain: *B. cereus* 6A5^T and VKM B-504^T. The fingerprints of these strains demonstrate practically identical electrophoretic patterns (Fig. 1). Minor differences in the intensity of the bands are most likely due to the specific features of the method of total DNA extraction. On the whole, the distribution of strains into closely related groups by Bc-REP PCR fingerprints did not always coincide with the data on their species affiliation. The same situation was also observed with other methods of typing such as MLEE, MLST, and AFLP. For instance, with MLST, a considerable share of the strains previously characterized as *B. cereus* were grouped alternately with *B. thuringiensis* strains [6].

PCR analysis of the presence of *cytK*, *hlyII*, and *hlyIIR* genes. PCR analysis of total DNA of bacilli under study has shown that the genes of cytotoxin K, hemolysin II, and its regulator, are widespread among members of the cereus group. Genes of hemolysin II and cytotoxin K were revealed in 56% and 61% of the analyzed strains, respectively. In the previously investigated collections of clinical and food poisoning-associated isolates, the *cytK* gene was found in 45% [13] and 37% [23] of strains, and the *hlyII* gene was found in 21% of strains [13]. Obviously, the high frequency of occurrence of the analyzed β-PFT is the result of the specific character of strain isolation sources. Thus, a considerable share of the microorganisms used in our work were isolated from soil and insects.

There is quite an expected pattern of occurrence frequency for the genes of hemolysin II and its regulator *hlyIIR*. All of the 15 strains carrying no *hlyII* gene also lack the *hlyIIR* gene, whereas 21 strains showed the presence of both genes simultaneously. This distribution of occurrence frequencies is due to the short distance (about 300 bp) between these genes, which, in turn, enhances the probability of their concurrent participation in recombinant events or joint horizontal transfer of both genes.

RFLP analysis of *cytK* and *hlyII*. The results of alignment of nucleotide sequences of the *hlyII* and *cytK* genes were used to reveal their polymorphic and conservative regions. Based on the nucleotide sequences of these regions, restriction endonucleases *Sau*3AI, *Rsa*I, and *Alu*I were selected for RFLP determination. Specific amplicons (calculated length, 813 bp) of the cytotoxin K genes were obtained for 23 (57.5%) of the 40 tested strains. In addition, eight variants of the *cytK* gene were analyzed on the basis of sequences available in GenBank. The sites of restriction endonucleases *Sau*3AI (six of six polymorphic sites) and *Alu*I (seven

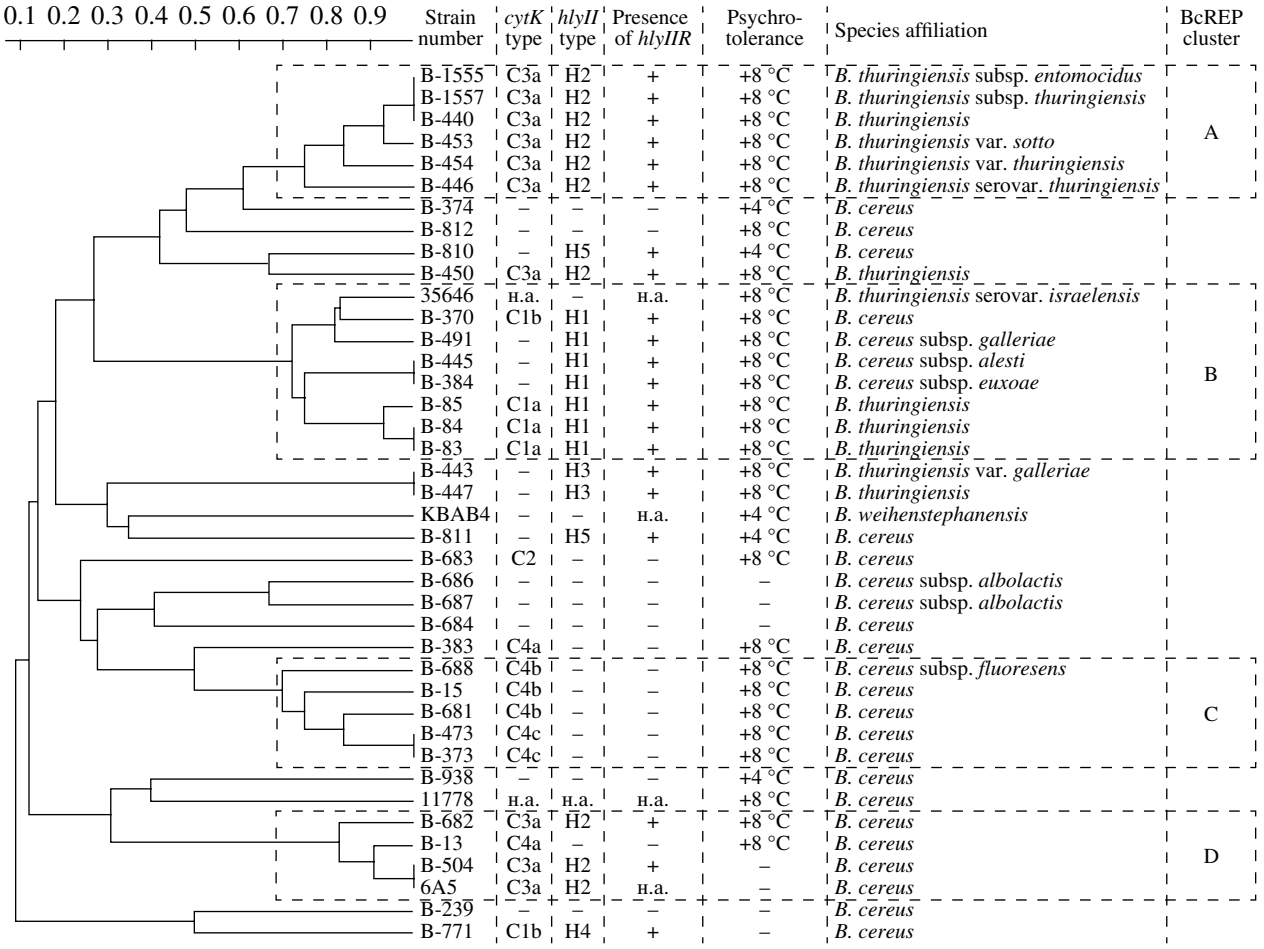

Fig. 2. Dendrogram of the cluster analysis of the group-specific fingerprints of strains. RFLP groups of the *cytK* and *hlyII* genes and the presence of the *hlyIIR* genetic determinant are given on the left, opposite the collection strain numbers. The minimal growth temperature for the corresponding strain is indicated in the "Psychrotolerance" column. Letters "A", "B", "C", and "D" indicate the isolated Bc-REP-PCR clusters; "–," absence of the gene; "+," presence of the gene; "n/a," presence of the gene was not analyzed.

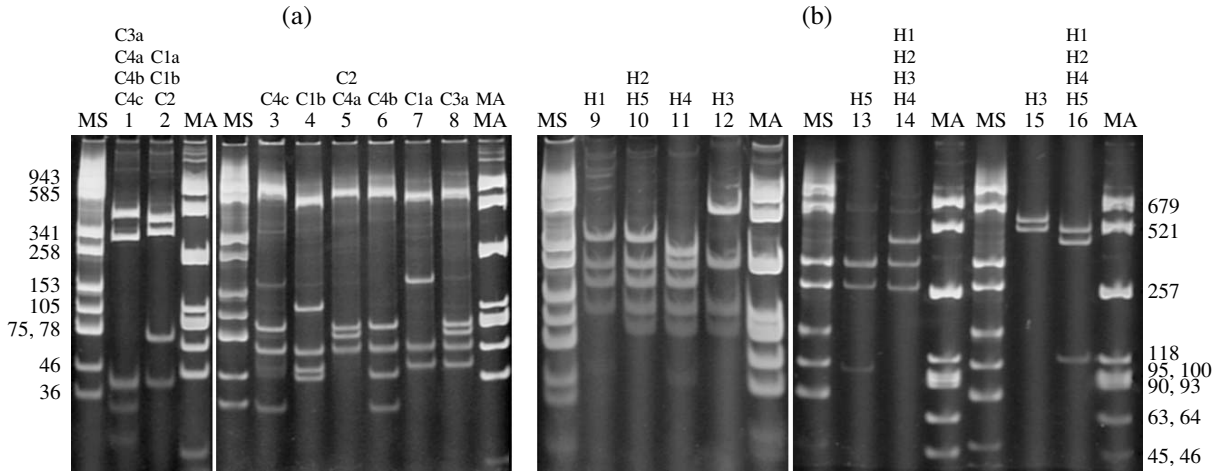

Fig. 3. Electrophoregrams of restriction profiles of the *cytK* and *hlyII* amplicons: A, *cytK* amplicons hydrolyzed by *Alu*I (tracks 1–2) and *Sau*3AI (3–8); B, *hlyII* amplicons hydrolyzed by *Alu*I (9–12), *Sau*3AI (13–14), and *Rsa*I (15–16). RFLP groups of genes with the same restriction profile are indicated above the track numbers. MS and MA, products of pUC18 hydrolysis by restriction endonucleases *Sau*3AI and *Alu*I, respectively. The sizes of DNA fragments in base pairs are given on the left and right of the figure.

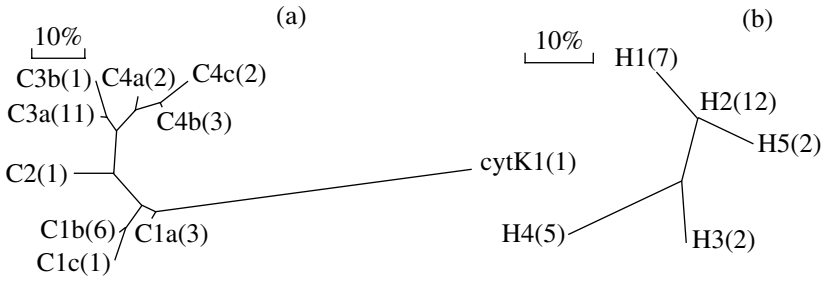

Fig. 4. Dendrograms of similarity of the *cytK* (a) and *hlyII* (b) genes based on the data of RFLP analysis. The number of strains with the specified gene type is given in brackets after group name. Strains that comprise the groups analyzed by PCR and RFLP are indicated in Fig. 2. Besides the above strains, groups include the *cytK* alleles: C1b: *cytK B. thuringiensis* strain 97-27, *cytK-2 B. cereus* ATCC 10987, *cytK-2 B. cereus* strain 23, *cytK-2 B. cereus* strain FM-1; C1c: *cytK B. cereus* strain E33L; C3a: *cytK-2 B. cereus* ATCC 14579^T; C3b: *cytK-2 B. cereus* strain 1230-88; *cytK1:cytK-1 B. cereus* strain 391-98, and the *hlyII* alleles: H2: *B. cereus* ATCC 14579^T, *B. thuringiensis* strain 97-27; H4: *B. anthracis* strain 'Ames Ancestor', *B. anthracis* strain Ames, *B. anthracis* strain Sterne, and *B. cereus* strain E33L.

of eight polymorphic sites) proved to be the most informative for the estimation of the *cytK* polymorphism (Fig. 3a). The location of a single RsaI site was found to be conservative. As a result, the analyzed strains were divided into ten groups by RFLP types of the *cytK* genes. Each group included 1–11 strains carrying the *cytK* genes (Fig. 4a). The genes previously identified as *cytK2* [13] were included in groups C1b, C3a, and C3b. No *cytK* genes similar to *cytK1* were found in any of the studied strains. The *cytK* genes of group C1a, which is the closest to the *cytK1* gene, differed from it in 8 of the 15 OTU. The genes of this group insignificantly differed from the previously described genes of the *cytK2* group C1b (in 1 OTU). Thus, all the tested genes should be considered as variants of cytotoxin K2, which is less dangerous for mammals. Similar results pointing at rare occurrence of *cytK1* alleles were obtained by analyzing the collection of 156 strains of *B. cereus sensu lato* [24]. Only 2 of the 89 strains carrying the *cytK* genes had *cytK1*.

Fragments of the *hlyII* gene were amplified using primers HF2 and HR2 in 56% of the strains. All PCR products were of the size corresponding to the calculated one: 955 bp (not shown). Based on the analysis of *hlyII* sequences from GenBank, seven more variants of the *hlyII* gene were used for investigation. The hydrolysis of amplicons by restriction endonucleases revealed two polymorphic sites of the three *Sau*3AI sites, one of the two *Rsa*I sites, and two of the seven *Alu*I sites (Fig. 3b). Based on the results of RFLP of the *hlyII* genes, 28 strains were combined into 5 groups. The resulting groups comprised 2–12 strains (Fig. 3b). Note that the genes of *B. anthracis* strains carrying a frameshift mutation and valid genes of *B. cereus* E33L and VKM B-771, the nucleotide sequences of which were determined in [25, 26], were clustered together.

Strains with the *hlyII*$^+$ and *cytK*$^+$ genotypes formed no distinct clusters (Fig. 2). The absence of correlation between phylogenetic positions of the strains and their clustering based on the presence and polymorphism of particular toxin genes and their regulators is typical of the representatives of the *B. cereus* group. For instance, the phylogenetic interrelation of strains based on MLST of conservative genes does not coincide with the clustering of strains based on the sequence of the *plcR* gene encoding the transcriptional activator of pathogenicity factors in microorganisms of the *B. cereus* group [6]. Similarly, the classes of "quorum sensing" compatibility of the *plcR–papR* pairs do not correspond with the phylogenetic position of strains determined by MLST [27].

Most groups of strains with similarity of over 70% had the same types of the *hlyII* gene and close types of the *cytK* gene (Fig. 2, clusters "A", "B", "C", and "D," and Fig. 4). However, Bc-REP clusters contain strains without these genes. For example, the *cytK* genes are absent in three strains of cluster "B" and the *hlyII* genes are absent in strain VKM B-13 of cluster "D" and ATCC 35646 of cluster "B". These findings most likely indicate the loss of genes in preceding generations, but suggest their presence in the ancestor of a group.

Detection and properties of psychrotolerant strains. The ability of strains to grow at 8°C was shown for 33 of the 40 microorganisms. The exceptions were VKM B-239, VKM B-504^T, VKM B-684, VKM B-686, VKM B-687, VKM B-771, and 6A5^T. The seven strains nonresistant to cold did not form a distinct group upon clustering. Five strains, including *B. weihenstephanensis* KBAB4, were able to grow at 4°C. The ability to grow at such temperatures is a characteristic feature of the species *B. weihenstephanensis* [5]. Psychrotolerant microorganisms were not grouped into a single cluster by the results of BcREP-fingerprinting (see Fig. 2). These strains proved to be able to produce hemolysins at 4°C. According to the PCR data, the gene of cytotoxin K is absent in all of the five strains, and two of them (VKM B-810 and VKM B-811) carry the gene of hemolysin II. Based on the RFLP analysis of the *hlyII* gene, these strains were classified in an individual cluster H5 (see Fig. 3b).

The applied method of clustering on the basis of BcREP-fingerprinting allowed us to isolate clusters containing phylogenetically close strains, which carried the same RFLP types of toxin genes *hlyII* and *cytK*. Strains of both *B. cereus* and *B. thuringiensis* were combined into the same BcREP-clusters, according to the results of typing. Presumably, this situation is due to the fact that determination of the species affiliation of the strains used in this work was based on classification by phenotypic characteristics. These characteristics, as is known, do not always reflect actual phylogenetic interrelations between microorganisms.

The gene of the hemolysin II regulator was revealed only in combination with the gene of hemolysin proper, which puts emphasis on their functional interrelation. According to the results of RFLP analysis, all strains of our collection carrying the *cytK* gene had a toxin variant which was less dangerous for mammals: cytotoxin K2. The results of the clustering of *cytK* genes based on the RFLP data allow distinct differentiation between *cytK1* and *cytK2*. Thus, the proposed test may be a potential basis for diagnostic detection of strains that may cause heavy poisoning induced by the *cytK1* gene product.

ACKNOWLEDGMENTS

The authors are grateful to Aleksey Sorokin for kindly providing the strains and to Zhanna Budarina for practical advice and discussion of results in the course of manuscript preparation.

The work was supported by Russian Foundation for Basic Research (grants 03-04-48623, 04-04-49693) and by a sub grant with Rutgers University, Office of Research and Sponsored Programs, under Sponsor Award No. 0853 from the Burroughs Welcome.

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