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Hemolytic and nonhemolytic enterotoxin genes of bacillus thuringiensis

This article presents new data about isolation of Bacillus group from different soil types and evaluation of enterotoxin genes frequency of isolated microorganism strains.
Keywords: Bacillus, enterotoxins, genes, hblA, nheA, cytK.

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Гены гемолитических и негемолитических энтеротоксинов bacillus thuringiensis

В статье приведены данные выделения представителей группы Bacillus из различных типов почвы и определения частоты встречаемости генов энтеротоксинов выделенных штаммов микроорганизмов.
Ключевые слова: Bacillus, энтеротоксины, гены, hblA, nheA, cytK.

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Гемолитикалық емес энтеротоксиндерінің гендері

Мақалада Bacillus тобының өкілдерін әр түрлі топырақ үлгілерінен бөліп алып, оларда энтеротоксин гендерінің кездесу жиіліктерін анықталған мәліметтер келтірілген.
Түйін сөздер: Bacillus, энтеротоксиндер, гендер, hblA, nheA, cytK.

The *Bacillus* genus consists of 77 species [1] and units the wide group of obligate aerobe or facultative anaerobe Gram-positive *chemoorganotrophic* rod-shaped *microorganisms which can produce thermostable endospores.*

According to «European Food Safety Authority», *Bacillus cereus* stands on the fourth place in a prevalence rate of foodborne illness causes [2]. The *Bacillus cereus group* is comprised of *Bacillus cereus sensu stricto*, famous for the ability to induce diarrheal and emetic syndromes, and also as a probiotic agent; *B. anthracis* which causes anthrax; *Bacillus mycoides* and *Bacillus pseudomycoides*, that can be characterized by typical *rhizoid growth; entomopathogenic B. thuringiensis* producing parasporal crystal toxins specifically active against some insect species; and psychrotolerant *Bacillus weihenstephanensis* [3]. Members of the *Bacillus* genus are found ubiquitously in nature and can be frequently found in soil. Genes responsible for specific traits of *B. anthracis* and *B. thuringiensis* are localized in macroplasmids. Nevertheless, one isolated strain of the *B. cereus* group is able to synthesize tens toxins, and responsible genes can be placed in a bacterial plasmid. Some of these toxins can induce severe foodborne illnesses in humans, and also may lead to fatal outcome.

One of the reasons prompting active study of toxins produced by *B. cereus* is the great significance of these microorganisms for human. Nowadays *B. thuringiensis* is commonly used for insecticide preparations production, which at the same time leads to extensive expansion of the microorganism in the environment. Other important issue is the bacteriologic contamination of industrially produced food, medicines and cosmetic treatments. In these fields *B. cereus* occurs to be the most common contaminator [4]. According to mentioned above, there is a need for determining the safety level of such microorganisms to the environments, animals

and humans. Within the framework of this aim examining of certain toxins produced by these bacteria is an essential element.

The *Bacillus* genus bacteria produce three types of toxins responsible for diarrheal foodborne diseases: *hemolytic enterotoxin (*Hbl*), non-hemolytic enterotoxin* (Nhe) and cytotoxin K (CytK) [1]. Considering that *B. thuringiensis* differs from *B. cereus* by the presence of plasmids encoding insecticide crystal toxins [4], B. cereus and B. Thuringiensis presents analogical frequency and expression of genes encoding these cytotoxins [3, 4]. Hbl and Nhe consist of three different protein components named L2, L1 and B, and NheA, NheB and NheC, respectively, while CytK represents single-component toxin [1]. The aim of the study was isolation of *B. cereus* group microorganisms from different countries and enterotoxin genes frequency evaluation of native isolates.

Materials and methods

As research objects were used samples from Kazakhstan (al-Farabi KazNU campus), Kenya (Kenya Shimba hills National Reserve and Tsavo *East National* Park) and Poland (Biebrza National Park).

To isolate B. thuringiensis a 10% soil solution (w/v) in 0.85% NaCl was shaken for 1 h (200–250 rpm) and then preheated in a water bath for 5 min at 72 °C to eliminate vegetative cells and to select the spores. For each serial dilution (10^{-1} to 10^{-4}), 100 µl were plated on MYP agar (Oxoid, Basingstoke, UK), a selective medium for isolating members of the B. cereus group, and incubated at 30 °C for 48 h. Those bacteria forming rough and dry colonies with a violet pink background surrounded by egg yolk precipitation on the MYP agar and with parasporal crystals observed under phase-contrast microscopy were identified as B. thuringiensis. The selected isolates were screened for haemolytic activity on Columbia Blood Agar (Oxoid) at 30 °C. B. cereus ATCC 14579 (American Type Culture Collection) were also included as reference strains for presence of the potential enterotoxin genes.

Olympus BX61 phase contrast microscope was used for bacterial crystals detecting.

Genomic DNA was extracted from overnight cultures grown in Luria–Bertani (LB) broth using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's instructions. The quantity and the purity of the extracted DNA were assessed using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

All strains were tested for the presence of the hblA and nheA genes with the pairs of primers designed by Hansen and Hendriksen [6].

The polymerase chain reaction (PCR) products of the cytK gene were detected using cytKF (GATAATATGACAATGTCTTTAAA) and cytKR (GGAGAGAAACCGCTATTTGT) primers (N. Michelet and J. Mahillon, unpublished data). The total DNA for PCR analysis was prepared from overnight cultures of the isolates in Luria–Bertani medium by the method of Bickley and Owen [7]. PCR amplifications were performed in a DNA HB-Cycler 022 (Helena BioSciences Com., UK) for 30 reaction cycles in a final volume of 25 mL containing 0.625 U Taq DNA polymerase, 250 ng of DNA, 200 mM deoxynucleotide mix, 1.5 mM MgCl2, and 0.5 mM concentration of each primer. The PCR conditions were as follows: a single denaturation step at 94-C for 3 min, denaturation of DNA template at 94-C for 1 min, annealing templates and oligonucleotide primers at 55^0C (hblA and nheA), at 55^0C (nheA) for 1 min, and extension of PCR products at 72-C for 1 min. An extra extension step was performed at 72-C for 10 min.

PCR products were separated on 1.0–1.5% (w/v) agarose gels ran in 1 x TBE, then photographed in UV light with the Gel Doc 2000 System (Bio-Rad Laboratories, Hercules, CA) and analyzed with the Quantity One PC version 4.1.1 program (Bio-Rad).

Results and discussion

200 bacteria strains of *Bacillus* genus were isolated from soil of Kazakhstan (KazNU named after al-Farabi campus), and provisionally named from AY1-1 to AY20-10 (AY series), 210 strains (SH, TE series) from Kenya (Kenya Shimba hills National Reserve and Tsavo *East National* Park) and named SH 1-1 – SH 10-10 and TE1-1 – TE11-10 and 87 strains from Poland National Park – IS5001 – IS5087 (IS series).

497 bacterial strains (200 from Kazakhstan, 87 from Poland and 210 from Kenya) were examined on the presents of enterotoxins.

Table 1 - Number of strains containing the enterotoxin genes

Strains genes	HblA	NheA	CytK
AY series	80	96	35
IS series	33	49	18
SH, TE series	144	199	179

Using PCR the presents of HblA gene was discovered in 40% of examined strains from Kazakhstan soil, in 38% examined strains from Poland soil and in 69% isolated from soil of Kenya. NheA and CytK enterotoxin genes were found approximately in 50% of *Bacillus* genus bacteria from AY and IS series, the major number of strains possessing these genes was from bacilli isolated from Kenya soil (95% and 85% respectively).

Obtained data correspond with data of other scientists [3, 8], which showed that the frequency of *Bacillus* enterotoxins varies geographically. For instance, in certain countries they induce less that 1% of all foodborne diseases, while in other countries – over 30%. *Bacillus cereus* is isolated from food comparatively often, what makes this bacteria species significant indicating test-organism for food industry. Food products, which are most exposed to risk of being contaminated are milk and meat products, vegetables, soups, condiments and, in particular, infant food. It is believed that HBL and NHE appear in a bowel of an ill person after eating the products contaminated with vegetative cells or spores of Bacillus cereus.

An extension of this work will involve further examination of the expression level of B. cereus enterotoxins and to clarify their possible implication for human health.

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