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Detection of Enterotoxic *Bacillus cereus* Producing Hemolytic and Non Hemolytic Enterotoxins by PCR Test

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

A b s t r a c t

Nine strains belonging to *Bacillus cereus* group has been isolated from food and environmental samples. Their taxonomic position was confirmed by RFLP analysis of 16S rRNA gene digested with *Taq*I. The detection of DNA sequences encoding the hemolysin BL complex and enterotoxin NHE, was studied in *Bacillus* sp. isolates. Set of primers was used to amplify fragment of *hblD* gene by PCR. For the detection of *nheB* gene a new primer set was developed which allowed to amplify 273 bp fragment from wide number of strains belonging to *B. cereus* group. The *hblD* gene was present in 7 out of 9 isolates whereas *nheB* gene occurred in all of them. Reference strains of *B. cereus* ŁOCK 0807, and *B. thuringiensis* NCAIM 01262 contained both genes. Strains of *B. subtilis* ATCC 6633 and *B. pumilus* LOCK 0814 do not contain both genes. Obtained results showed that *B. thuringiensis* NCAIM 01262 contains both genes and therefore may be harmful for human beings.

K e y w o r d s: enterotoxin BL, enterotoxin NHE, *Bacillus cereus* group, PCR

Introduction

Bacillus anthracis, Bacillus cereus and *Bacillus thuringiensis* are members of the *Bacillus cereus* group of bacteria sharing common properties and strong genetic similarity especially in the sequence of 16S rRNA, the number of rRNA operons, their organization, localization and ability to produce numerous toxins responsible for pathogenicity and food poisonings. *B. anthracis* causes the acute fatal disease, anthrax, and is widely recognised as powerful biological weapon due to its high toxicity for human beings. *B. thuringiensis* is a well known producer of crystalline intracellular proteins called δ-endotoxins, toxic to wide number of insect larvae belonging to *Diptera* and *Coeloptera*. For this reason cells of *B. thuringiensis* are widely used as biological pesticide and its genes coding for δ-endotoxins were used for the construction of pest resistant transgenic crop plants. *B. cereus* is a very common soil bacterium which may contaminate starchy food products and raw materials causing diarheal and emetic food poisonings. Formation of thermoresistant spores by *Bacillus cereus* may also cause sterility problems in ready to eat dishes consisting of boiled rice, noodles, potato and other starch-containing food products.

Advent of modern genetics brought a lot of information on chromosome organization of the members of *B. cereus* group. So far two *B. anthracis* strains Ames and Sterne (Read *et al*., 2003; Brettin *et al*., 2004c), three *B. cereus* strains ATCC 14579 (Ivanova *et al*., 2003), ATCC 10987 (Rasko *et al*., 2004) and ZK (Brettin *et al*., 2004b) as well as one *B. thuringiensis* serovar *konkukian* strain 97–27 (Brettin *et al*., 2004a) were sequenced and the data obtained were disseminated to the public through computer databases. This

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makes ideal situation for comparative genomics *in silico* to study distribution of disease related genes in all members of that group. From the viewpoint of food poisonings caused by *B. cereus*, presence of two gene clusters responsible for the production of hemolytic HBL and nonhemolytic NHE enterotoxins are very important. Search of computer databases revealed presence of non hemolytic enterotoxin gene cluster *nheABC* in all sequenced members of *B. cereus* group showing that even recognized as "non toxic" *B. thuringiensis* serovar *konkukian* str 97–27 can cause food poisonings. Gene cluster responsible for the production of hemolysin BL *hblCDA* was found only in one strain of *B. cereus* ATCC 14579 and in *B. thuringiensis* serovar *konkukian* str 97–27. This findings also confirmed possible toxicity of strain known as "harmless". Therefore detection of enterotoxin production potential of strains belonging to the *B. cereus* group is very important for the epidemiology of food poisonings. Available methods of detection of enterotoxic *B. cereus* group were as follows: 1 – blood cell hemolysis on Columbia Agar medium, 2 – immunological tests, *Bacillus* diarrheal enterotoxin visual immunoassay (BDE kit Tecra), (BCET-RPLA kit Oxoid), 3 – PCR test. The *B. cereus* enterotoxin test kit RPLA, Oxoid is specific to the L_2 component of the hemolysin BL complex whereas BDE kit Tecra detects two non toxic proteins of 40 and 41 kDa characteristic to enterotoxic strains (Beecher and Wong, 1994). Detection of both enterotoxins with PCR based technique has been developed by Hansen and Hendriksen (2001) who proposed sets of primers for individual genes of *hblCDA* and *nheABC* operons. Mantynen and Lindstrom (1998) and Neil *et al*. (2003) used PCR technique for detection of *hblCDA* operons. Comparison of immunological methods for detection of enterotoxin producing strains with PCR-based technique showed good correlation of obtained results and therefore both techniques can be equally reliable and repleaceable (Mantynen and Lindstrom, 1998). These authors have also shown that presence of enterotoxin coding genes are not characteristic for the genus *B. cereus* but also can be found in *Bacillus mycoides*, *Bacillus pasteurii*, *Bacillus smithii* and *Bacillus thuringiensis*. However among 50 strains of *B. cereus*, only 26 contained enterotoxin coding genes suggesting that this feature is strain specific. Presence of enterotoxin genes in non cereus group, *Bacillus pasteurii* and *Bacillus smithii*, may indicate that ability to produce the same enterotoxins can be found in other species belonging to the genera *Bacillus*.

The aim of the present study was to develop rapid and accurate diagnostic tests for detecting *Bacillus cereus* group genes responsible for the production of enterotoxins. In order to determine phylogenetic position of studied strains we performed 16S rRNA gene RFLP analysis.

Experimental

Materials and Methods

Bacterial strains used in this study. Bacterial strains applied in this study (*Bacillus* sp. 1 – sp. 9) were isolated from different food products according to the method described in the PN-EN ISO 7132 using MYP selective medium (Oxoid). *B. cereus* ŁOCK 0807, *B. thuringiensis* NCAIM 01262, *B. pumilus* ŁOCK 0814 and *B. subtilis* ATCC 6633 were used as reference strains. They originated from Culture Collection of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź.

DNA preparation. Chromosomal DNA was isolated according to the modified method of Marmur (1961). Additional lysozyme treatment of digested cell suspension with proteinase K was the major modification of original method.

PCR amplification of 16S rRNA, *hblD* and *nheB* fragments. Primer sequences for amplification of *hblD*, *nheB* and 16S rRNA fragments were derived from data records published in NCBI Database (Table I). Amplification of *hblD* fragment (430 bp) was performed in the following manner. About 20 ng of DNA template, 20 pmol of primer hblDF, 20 pmol of primer hblDR, 12.5 µl Red-Taq ReadyMix DNA polymerase (Sigma-Aldrich) were mixed together and supplemented with PCR grade water to a total volume of 25 ml. The amplification procedure consisted of one cycle of 2 min at 94°C, followed by 35 cycles for 30 sec at 94°C,

Table I
Primers used for PCR amplification of 16S-rRNA, *hblD*, *nheB* fragments

DNA sequence	Name	Primer	Sequence source
16S rRNA	FRNA RRNA	5'-AGAGTTTGATCCTGGCTCAGGA-3' 5'-GGAGGTGATCCAGCCGC-3'	AE016877
hblD	hblDF hblDR	5'-AATCAAGAGCTGTCACGAAT-3' 5'-CACCAATTGACCATGCTAAT-3'	Hansen and Hendriksen (2001)
nheB	nheBF nheBR	5'-ATGACAAAAAAACCTTATAAAGTAATG-3' 5'-ATTTCCAAAGTTAACATTACCTTGT-3'	This work

30 sec at 48°C and 90 sec at 72°C with final extension cycle for 2 min at 72°C was performed using Uno II thermocycler, Biometra, with tube lid heating block set for 105°C. No overlay oil was added to the tubes. The reaction mix for amplification of *nheB* fragment (273 bp) was the same except of primers replaced by nheBF and nheBR in the concentration of 20 pmol each. The amplification procedure for *nheB* fragment consisted of one cycle of 2 min at 94°C, followed by 35 cycles for 30 sec at 94°C, 30 sec at 50°C and 90 sec at 72°C with final extension cycle for 2 min at 72°C. In case of 16S rRNA gene amplification the reaction mix was the same but primers were replaced by FRNA and RRNA in the concentration of 20 pmol each. The amplification procedure for 16S rRNA fragment consisted of one cycle of 2 min at 94°C, followed by 35 cycles for 1 min at 94°C, 1 min at 55°C and 3 min at 72°C with final extension cycle for 2 min at 72°C.

RFLP analysis. The 16S rRNA amplification fragments were digested with *Taq*I (MBI Fermentas) for 1 h at 65°C according to the product instruction. The digested PCR products were run at 60V for 4 h in 2% (w/v) agarose gel (0.5 TBE buffer containing 0.5 µg/ml ethidium bromide by using gel electrophoresis apparatus (Biotec Fisher). Gells placed on UV transiluminator were photographed with digital camera through yellow filter.

Agarose gel analysis of PCR products. PCR products of *hblD* and *nheA* fragments after amplification were analysed on 1% (w/v) agarose gel in 0.5 TBE buffer containing 0.5 µg/ml ethidium bromide. Gels were run at 60 V for 3 h and photographed as described above.

Results and Discussion

RFLP analysis of 16S rRNA amplicons of bacterial isolates and reference strains (Fig. 1) confirmed previous findings from classical diagnostic procedure (PN-EN ISO 7132) that all *Bacillus* sp. 1–9 isolates belongs to the cereus group. Profile comparison of lane 1 and 2 in Fig. 1 showed that RFLP patterns for the

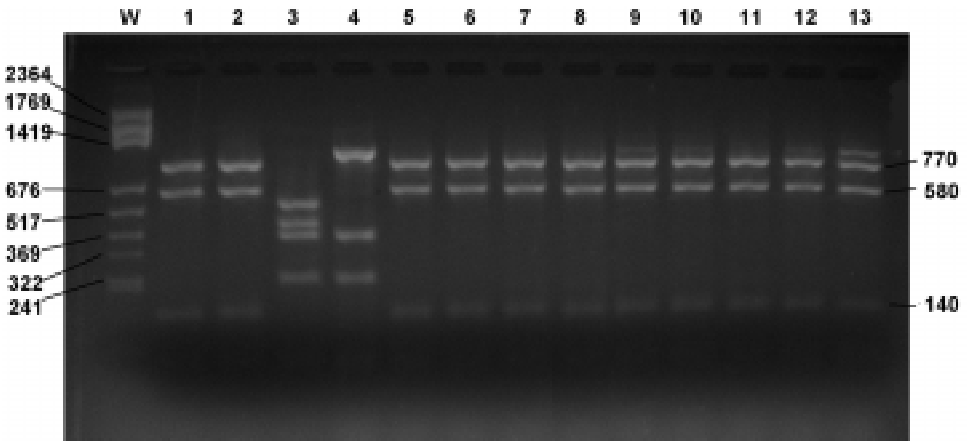

Fig. 1. RFLP profiles of 16S rRNA gene amplicons digested with *Taq*I.

W – DNA size marker; lanes 1– *B. thuringiensis* NCAIM 01262; 2 – *B. cereus* ŁOCK 0807; 3 – *B. subtilis* ATCC 6633; 4 – *B. pumilus* ŁOCK 0814; 5 – *Bacillus* sp. 1; 6 – *Bacillus* sp. 2; 7 – *Bacillus* sp. 3; 8 – *Bacillus* sp. 4; 9 – *Bacillus* sp. 5; 10 – *Bacillus* sp. 6; 11 – *Bacillus* sp. 7; 12 – *Bacillus* sp. 8; 13 – *Bacillus* sp. 9.

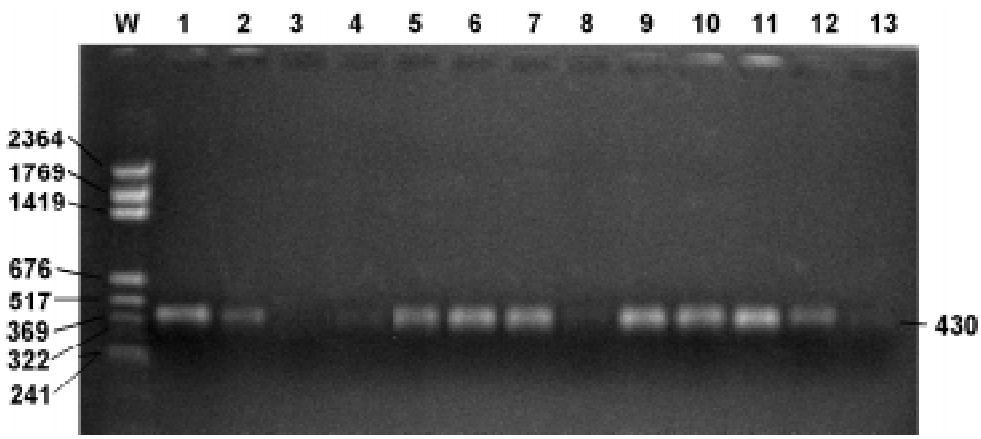

Fig. 2. Electrophoregram of *hblD* gene fragment amplicons.

W – DNA size marker; lanes 1 – *B. thuringiensis* NCAIM 01262; 2 – *B. cereus* ŁOCK 0807; 3 – *B. subtilis* ATCC 6633; 4 – *B. pumilus* ŁOCK 0814; 5 – *Bacillus* sp. 1; 6 – *Bacillus* sp. 2; 7 – *Bacillus* sp. 3; 8 – *Bacillus* sp. 4; 9 – *Bacillus* sp. 5; 10 – *Bacillus* sp. 6; 11 – *Bacillus* sp. 7; 12 – *Bacillus* sp. 8; 13 – *Bacillus* sp. 9.

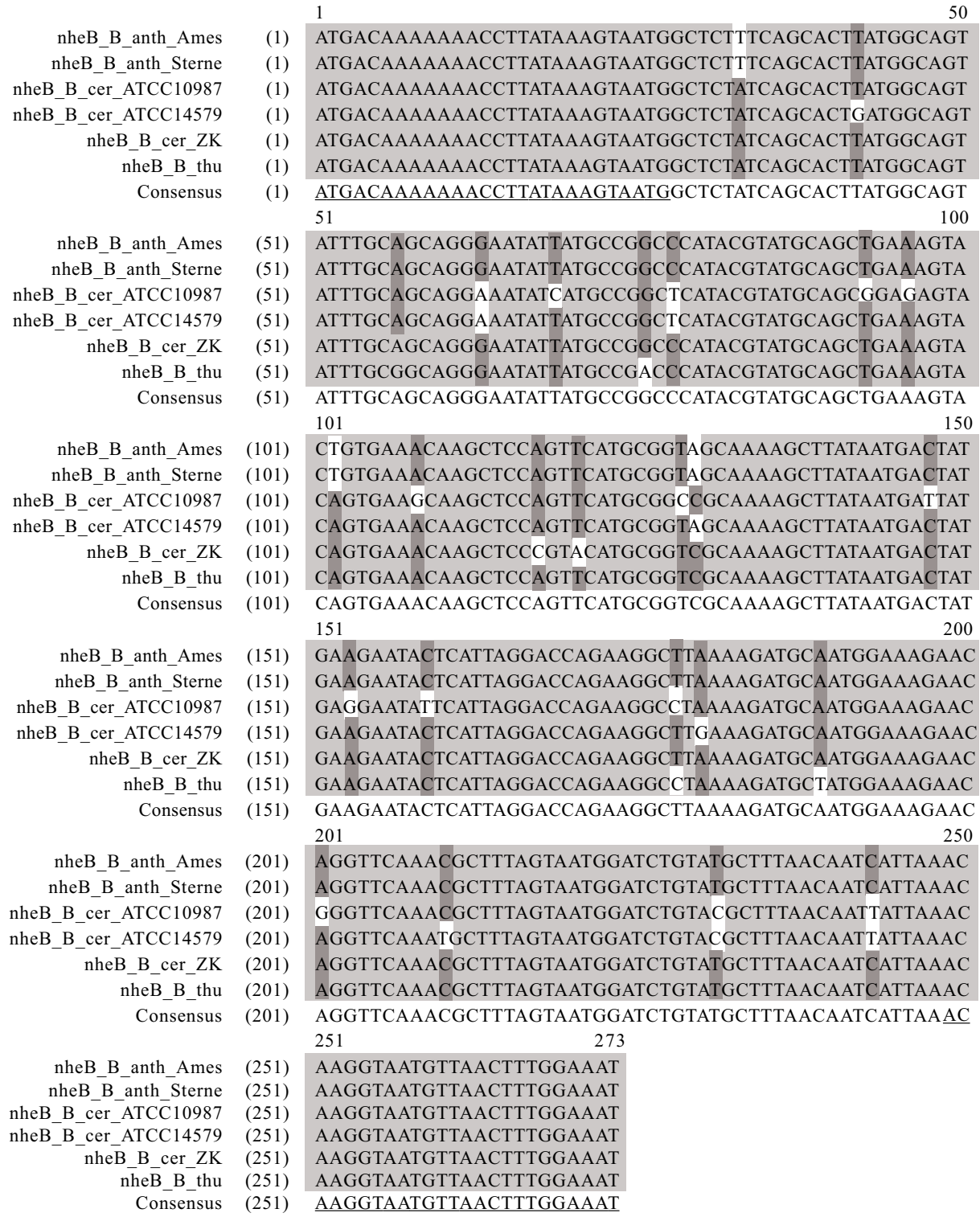

Fig. 3. Alignment of 273 bp long fragment of *nheB* DNA sequence from fully sequenced chromosomes of *B. anthracis* strain Ames (AE016879). *B. anthracis* strain Sterne (AE017225), *B. cereus* ATCC 10987 (AE017994), *B. cereus* ATCC 14579 (AE016877), *B. cereus* ZK (CP000001) and *B. thuringiensis* serovar *konkukian* strain 97–27 (AE017355). Underlined sequences represents universal primers designed in this work for detection of *nheB* gene.

reference strains *B. thuringiensis* NCAIM 01262 and *B. cereus* ŁOCK 0807 are the same and therefore, these species can not be distinguish with this method. This finding has been confirmed by computer analysis of published sequences of 16S rRNA genes from all four members of cereus group (*B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis*), showing that *Taq*I recognition sites within the region of that gene resides at 63, 201 and 972 bp positions. Taking into account that amplified fragment is 7 bp shorter from the front and

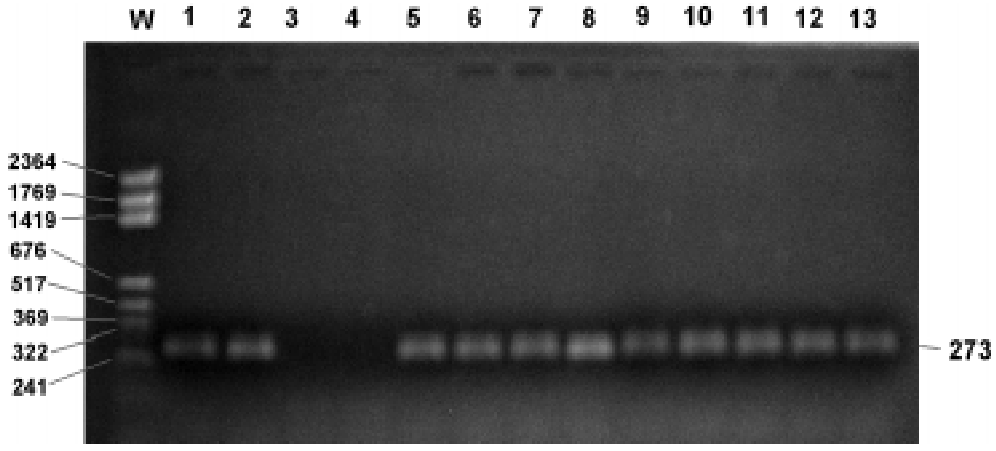

Fig. 4. Electrophoregram of *nheB* gene fragment amplicons.
W – DNA size marker; lanes 1– *B. thuringiensis* NCAIM 01262; 2 – *B. cereus* ŁOCK 0807; 3 – *B. subtilis* ATCC 6633; 4 – *B. pumilus* ŁOCK 0814; 5 – *Bacillus* sp. 1; 6 – *Bacillus* sp. 2; 7 – *Bacillus* sp. 3; 8 – *Bacillus* sp. 4; 9 – *Bacillus* sp. 5; 10 – *Bacillus* sp. 6; 11 – *Bacillus* sp. 7; 12 – *Bacillus* sp. 8; 13 – *Bacillus* sp. 9.

4 bp from the end of complete 16S rRNA gene, resulting *Taq*I digestion products should have the following size: 56, 138, 579, 771 bp. Obtained results confirmed usefulness of applied RFLP analysis for genetic confirmation of taxonomic position of isolated strains.

Application of primers developed by Hansen and Hendriksen (2001) allowed to dectect *hblD* gene (Fig. 2). The obtained results showed that in seven isolates of *Bacillus* sp. 1–3 and sp. 4–8 as well as in both reference strains *B. thuringiensis* NCAIM 01262 and *B. cereus* ŁOCK 0807 this gene was present. Strains of *Bacillus* sp. 4 and 9 showed presence of *hblD* related bands (430 bp) with very low intensity what may be interpreted as lack of this gene. However this result can be obtained because the incompatibility of primers with the polymorphic DNA of *hbl*D gene in that strains. The other reference strains of *B. subtilis* ATCC 6633 and *B. pumilus* ŁOCK 0814 did not have that gene. Presence of *hblD* gene in the reference strain of *B. thuringiensis* NCAIM 01262 shows that this organism can be potentially harmful for human beings what is in disagreement with the common opinion of microbiologists.

Polymorphism of *nheB* genes found in *B. anthracis* (strains Ames and Sterne), *B. cereus* (strains ATCC 10987, ATCC 14579, ZK) and *B. thuringiensis* serovar *konkukian* str. 94–27 analysed *in silico* showed that primers designed by Hansen and Hendriksen (2001) are not 100% identical to the corresponding regions of that genes. Similarity of forward primer varied from 94.7 to 100% and reverse primer from 88.2 to 100%. This observation lead us to the conclusion that such primers can not be used for reliable PCR-based detection of *nheB* gene in the wide number of bacterial species. Therefore, an attempt has been made to design our own set of universal primers with 100% similarity to the corresponding regions of *nheB* genes of all already sequenced *Bacillus* strains. Figure 3 shows alignment of 273 bp long fragment of *nheB* DNA sequence from genomes of *Bacillus* strains belonging to the *B. cereus* group. PCR reaction made with this primers for 9 *B. cereus* isolates and 4 reference strains from culture collection revealed presence of characteristic 273 bp long amplicons for all isolates of *Bacillus* sp. 1–9, *B. cereus* ŁOCK 0807 and *B. thuringiensis* NCAIM 01262. Strains of *B. pumilus* ŁOCK 0814 and *B. subtilis* ATCC 6633 were deprived of *nheB* gene (Fig. 4). Obtained results confirmed observations from *in silico* analysis of *B. cereus* group genomes that *nheABC* operon is characteristic for that group of organisms. This finding also confirmed previous statement that *B. thuringiensis* can be harmful for human being and its use for the production of biological pesticides may create biological hazard.

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