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PREVALENCE, VIRULENCE GENES, AND GENETIC DIVERSITY OF *BACILLUS CEREUS* ISOLATED FROM CONVENIENCE FOOD

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ABSTRACT

Background. In times when there is a growing interest in ready-to-eat food (RTEF), the presence of potentially pathogenic bacteria, including the toxigenic psychrotolerant bacilli from the *B. cereus* group, on this type of carrier may pose a real threat to the health of consumers. A significant part of RTEF is represented by vegetable products and food products made with them. The increased production of convenience foods has resulted in their international turnover growing. When coupled with a rising percentage of persons from risk groups (YOPI), including the elderly or immunocompromised, this may mean increased health risks posed by the so-called "novel pathogens", like the toxigenic psychrotolerant *B. cereus* sensu lato.

Materials and methods. Food samples were analyzed for the presence and count of putative *B. cereus* according to the Polish Standard PN-EN ISO 7932:2005. All genetic analyses were conducted using a qualitative real-time PCR.

Results. The presence of *B. cereus* sensu lato was confirmed in 130 out of the 192 samples of convenience foods, at contamination levels ranging from 1.65 to 3.32 log CFU/g. Among the strains confirmed to belong to the *B. cereus* group, 23 were identified as emetic *B. cereus*. The analysis of each strain's ability to grow at temp. 4–10°C demonstrated that 4.9% and 12.7% of the isolates were able to grow at 4°C and 6°C, respectively. In turn, 15.2% were able to grow at 8°C, and 36.3% at 10°C. None of the psychrotrophic strains possessed genes typical of *B. weihenstephanensis.* The group of psychrotrophic *B. cereus* included potentially toxigenic strains being carriers of genes that determine the synthesis of the following toxins: NHE, HBL, CytK, and cereulide. Some of them were potent enough to produce more than one toxin.

Conclusions. The analyses conducted in this study demonstrate that the psychrotolerant strains of *B. cereus* (including the toxigenic ones) are frequent microbiological contaminants of RTEF products offered in retail. The presence of emetic strains from the *B. cereus* group, which are able to grow in a wide range of temperatures and produce enterotoxins and enzymes with the characteristics of toxins, in ready-to-eat foods may pose a real threat to consumer health.

Keywords: ready-to-eat food, psychrotolerant bacilli, emetic strains, enterotoxins

INTRODUCTION

Bacilli of *B. cereus* sensu lato are widespread in the natural environment (i.e., in soil, wastewater, water, and dust) and in fertilizers and feeds. As primary or secondary contaminants, they may be found in food products of various origins and various degrees of processing (Lindbäck et al., 2004), including mainly those of plant origin (Subramanian et al., 2006).

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The *B. cereus* (*B. cereus* sensu lato) group is today distinguished within the genus *Bacillus*. It includes genetically closely related species *Bacillus anthracis*, *B. cereus* sensu stricto, *B. cytotoxicus* sp. novel (Guinebretiere et al., 2013), *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, psychrotolerant *B. weihenstephanensis* – able to grow at low temperatures (below 7°C) and unable to grow at 43°C – and *B. toyonensis* (Jiménez et al., 2013) as its most prominent members. In addition, strains of *B. weihenstephanensis* distinguish themselves in the *B. cereus* group with a unique sequence of the *cspA* gene encoding for cold shock protein and with a 16S rDNA sequence being typical of psychrophiles (Thorsen et al., 2006). Furthermore, three *B. cereus* group strains have been proposed as representing novel species, i.e., *B. gaemokensis* (Jung et al., 2010), *B. manliponensi*s (Jung et al., 2011), and *B. bingmayongensis* (Liu et al., 2014), but have not yet been validly published. *B. cereus* bacteria may induce food poisoning by the synthesizing of non-hemolytic and hemolytic complexes of enterotoxins, cytotoxin K (CytK), and an emetic toxin (cereulide). This study aimed to determine the prevalence and counts of *B. cereus* sensu lato in RTEF products requiring cold preservation, to identify the ability of these bacteria to grow at temperature 4–10°C, and to determine the toxigenic potential of psychrotolerant strains of *B. cereus* sensu lato.

MATERIALS AND METHODS

Materials

The experimental material included 192 samples of convenience food products requiring cold storage, i.e., green salads made of salad vegetables (lettuce, onions, tomatoes) with chicken and feta cheese (64), unpasteurized fruit and vegetable juices (34), minimally processed vegetables (46), mayonnaise (28), and sushi (20).

Isolation of strains

The quantitative detection of *B. cereus* was performed according to the Polish Standard (PN-EN ISO 7932:2005). In brief, 10 mL or g of the sample was mixed and homogenized with 90 mL of buffered peptone water (Scharlau, Spain). The samples were then serially diluted with 9 mL of 0.9% sterile saline (1:10 dilution) and thoroughly mixed. Next, the cultures were streaked in 0.1 mL portions on duplicate Mannitol Egg Yolk-Polymyxin (MEYP) Agar plates (Selective media; BTL, Poland) and incubated at 30°C for 24 h. The characteristics considered during food analyses with respect to suspected *B. cereus* were evaluated on the MEYP medium (capability for mannitol degradation and lecithinase production) and Sheep Blood Agar (7% defibrinated sheep blood; Scharlau, Spain) (hemolysin production – type β hemolysis). Colonies with pink sparkle were picked for further biochemical identification using the *B. cereus* biochemistry. The morphology of the cultures was also checked microscopically. The isolates which were β-hemolysin positive, rod-shaped, and had central or subterminal spores were considered to belong to *B. cereus* sensu lato. They were transferred to cryo-tubes containing TSB with 15% (v/v) glycerol and stored at –80°C until required.

Capability to grow in a temperature range of 4–10°C and at 43°C

Strains of *B. cereus* sensu lato were cultured at temperatures of 4, 6, 8, 10, and 43°C in a liquid TSB medium (BTL, Poland). Culture growth was evaluated macroscopically after 24 h at a temperature of 30°C, after 24–48 h at 43°C, and after 10 days at temperatures of 4–10°C. Positive cultures were checked for purity by inoculating them onto the MEYP medium (BTL, Poland).

Isolation of genomic DNA and methods of genetic analysis

Bacterial DNA was isolated from 1 mL of 20-h bacterial cultures of *B. cereus* sensu lato strains grown on the TSB medium (Oxoid, USA) using a commercial Genomic Mini AX Bacteria kit (A&A Biotechnology, Poland). The qualitative analysis of the isolated genetic material was conducted with a spectrophotometer (NanoDrop 1000, NanoDrop Technologies, Gibraltar). Until analysis, the DNA samples were stored at a temperature of –32°C. The genetic analyses included a determination of the analyzed strains' affiliation to *B. cereus* group (detection of a specific sequence of the 16S rDNA gene) and the presence of specified toxin-encoding genes (*ces* gene), *hblA*, *hblC*, *hblD* genes, *nheA*, *nheB*, *nheC* genes and *cytK* gene in the psychrotolerant *B. cereus* sensu lato. The analyses

Table 1. Sequences of primers used in the identification of *B. weihenstephanensis*

Determined genes	Amplicon size	Primer	Primer sequence 5' 3'	Reference
16S rDNA (p)	132bp	16SpF	CAAGGCTGAAACTCAAAGGA	Stenfors Arnesen et al. (2007)
		16SpR	GAGAAGCTCTATCTCTAGA	
16S rDNA (m)	249bp	16SmF	ATAACATTTTGAACCGCATG	Stenfors Arnesen et al. (2007)
		16SmR	CTTCATCACTCACGCGGC-3	
cspA	160bp	CspAF	GAGGAAATAATTATGACAGTT	Francis et al. (1998)
		CspAR	CTTYTTGCCTTCTTCTAA	

were carried out with reference to strains of *B. cereus* sensu lato: *B. cereus* F4810/72 – *ces* gene and16S rDNA gene, *B. thuringensis* DSM 5725 – *hblA*, *hblC hblD*, *cytK* genes, *B. thuringensis* PCM 2517 – *nheA*, *nheB* and *nheC* genes, and *B. weihenstephanensis* DSM 11821 – 16S rDNA (p), 16S rDNA (m) and *cspA* genes, from which the genetic material was isolated as previously described. The presence of cytotoxin K (CytK) genes was determined using a degenerate primer, which allowed the detection of two types of cytotoxin K: CytK-1 and CytK-2. All analyses were conducted with the qualitative real-time PCR (LightCycler 480 Multiwell Plate 96, Roche, Switzerland). The melting temperatures (Tm) of individual reaction products were compared with those obtained for the standard *B. cereus* strains possessing the analyzed genes (Table 1). The comparative analyses were carried out using LightCycler Software (Version 1.5.0 SP3). The presence of the real-time PCR product with a T_m value identical to that of the standard was considered a positive result. The primers used in this study and the conditions of particular reactions are provided in Table 1 and Table 2. Genetic differentiation analysis was carried out using the Random Amplified Polymorphic DNA method (RAPD-PCR). Strains of *B. cereus* were differentiated using the R3 GCGATCCCCA primer (Ghelardi, et al., 2007). The RAPD--PCR reaction was conducted in 25 µL of the reaction mixture containing 500 mM KCl (Eppendorf, Germany), 100 mM Tris-HCl (pH 8.3 at 25°C), 1.25 mM $MgCl_2$ (Eppendorf, Germany), 0.3 mM dNTP (Roche, Switzerland), 20 pmol/µL primer (Genomed, Poland), 1 U Taq DNA polymerase (Eppendorf, Germany), and 20 ng DNA template. The thermal profile used in the reaction consisted of 35 cycles, including: 60 s/94°C, 60 s/37°C, and 60 s/72°C (Mastercycler Gradient Eppendorf, Germany). The amplification products were separated electrophoretically in a 2.0% agarose gel (Agarose LE, Roche, Switzerland) with ethidium bromide (0.5 µL/mL) (Bio-Rad, USA). The results of the electrophoretic separation were visualized in UV rays in a GelDoc apparatus (Bio-Rad, USA). The strain affinity degree was determined based on the cluster analysis of the obtained amplification profiles (RAPD) using the Unweighted Pair Group Method with the Arithmetic Mean (UPGMA) method in Bio-Gene software (Vilber-Lourmat, France), with a Dice coefficient of 3.0%.

Table 2. Qualitative real-time PCR conditions used in the identification of *B. weihenstephanensis*

Determined genes	Components of reaction	Primer hybridization temperature
16 S rDNA (p)	LightCycler 480 SYBR Green I Master, 5pM each primers	53°C
cspA	LightCycler 480 SYBR Green I Master, 5pM each primers	52°C

RESULTS

The presence of *B. cereus* sensu lato was confirmed in 130 out of the 192 analyzed food samples (67.8%) at contamination levels ranging from 1.65 to 3.32 log CFU/g, and its 204 isolates were obtained (Table 3). The prevalence of *B. cereus* sensu lato in the analyzed convenience foods reached 67.8%. It was most frequently isolated from green salads (76.6%), and minimally processed vegetables (69.6%). Affiliation to the *B. cereus* group and the capability to produce emetic toxin were determined with the duplex real-time PCR technique based on the presence of 16S rDNA gene sequence (288bp) specific for the *B. cereus* group and *ces* gene sequence (188bp). All isolated strains were confirmed to belong to the *B. cereus* group. Among them, 23 strains were identified as emetic strains of *B. cereus* (Table 3). Among the 204 strains analyzed, 4.9% and 12.7% were able to grow at temperatures of 4°C and 6°C, respectively (Table 4). In turn, 15.2% of them were able to grow at a temperature of 8°C, 36.3% at a temperature of 10°C, and 86.3% at a temperature of 43°C. The analysis of *B. cereus* growth at various temperatures revealed that psychrotrophic traits were most frequently displayed by the strains isolated from sushi. The study results lead to the conclusion that only the non-emetic strains were able to grow at 4°C. The minimal temperature ensuring the growth of emetic

Table 3. Prevalence of *B. cereus* sensu lato in convenience food

Sample type	Number of positive samples %	Mean count log CFU/g	Number of isolated *B. cereus* / number confirmed *B. cereus*	Number of emetic *B. cereus*
Green salads	49/64 (76.6)	2.26 ±0.06	68/68	10
Fruit and vegetable juices	20/34 (58.8)	1.65 ±0.33	32/32	0
Vegetables	32/46 (69.6)	3.11 ±0.22	45/45	2
Mayonnaise salads	18/28 (64.3)	3.32 ±0.13	37/37	4
Sushi	11/20 (55.0)	3.26 ±0.13	22/22	7
Σ	130/192 (67.8)		204/204	23

Values are means ±standard deviations (SD).

Table 4. Distribution of enterotoxin and emetic toxin genes in psychrotolerant *B. cereus* senso lato isolated from convenience food

	Number of strains carrying toxins								
Product (number of strains)	HBL + NHE	HBL+ CytK	NHE + CytK	HBL + NHE + CytK	HBL	NHE	CytK	Cer + NHE	Cer
Green salads (23)	1	6	2	4	2	6	2	0	0
Fruit and vegetable juices (7)	0	0	2	0	1	4	0	0	0
Vegetables (12)	0	0	3	0	2	6	1	0	0
Mayonnaise salads (17)	2	1	1	1	2	8	2	0	0
Sushi (12)	1	3	1	1	4		2	0	0
Σ (71)	4	10	9	6	11	24	7	0	0

strains turned out to be 6°C. This was particularly true for the isolates from sushi. In our study, nearly 36.3% of the isolated *B. cereus* sensu lato strains grew at temperatures ≤10°C. The simultaneous presence of a 16S rDNA gene sequence specific for the psychrotrophic strains and of the *cspA* gene encoding for the cold shock protein in the genome of a strain indicates its affiliation to the *B. weihenstephanensis* species. Interestingly, the PCR did not confirm affiliation to this species (lack of *csp*A gene) for 6 strains with growth temperatures typical of *B. weihenstephanensis* (growth at temperatures of 4–7°C, growth disability at 43°C). The analyses demonstrated the presence of the 16S rDNA gene sequence typical of the psychrotrophic strains in all analyzed strains from the *B. cereus* group (both the emetic and non-emetic ones). In contrast, the *csp*A gene homolog was not detected in any of the strains. When confronting the results of the determinations of gene sequences responsible for the psychrotrophic character of the strains with strain capability to grow at low temperatures, it may be concluded that 4.9% of the strains (10/204) grew at 4°C even though they possessed the 16S rDNA (p) gene sequence. None of the isolated strains were representative of *B. weihenstephanensis*.

The presence of genes of HBL, NHE, and CytK enterotoxins was analyzed in 71 strains. Because sets of three genes are required for HBL and NHE synthesis, i.e., *hblACD* and *nheABC* respectively, the presence of these genes was treated jointly when determining a strain's potential for enterotoxin production. With a significant prevalence of *B. cereus* sensu lato strains being carriers of individual genes responsible for the synthesis of enterotoxins, the simultaneous presence of three DNA sequences encoding three components of the NHE toxin (*nheA*, *nheB*, *nheC*) was detected in 43 strains of *B. cereus*, i.e., in 60.6% of the analyzed strains (Table 4). The potential for HBL toxin production was revealed in 31 strains. Some of the psychrotolerant strains of *B. cereus* were putative producers of more than one toxin (Table 4). Finally, 32 strains were shown to be capable of producing cytotoxin K. The analysis of the genetic profiles of strains from different environments with different enterotoxic potential allowed 2 groups (clusters) sharing similar RAPD formulas to be identified – determined based on the Dice coefficient (3%) – at the level of ~45%. Based on the genetic similarity of the strains at >75%, 17 types of RAPD were grouped, and the groups contained from 2 to 8 strains. Cluster 1 was formed by 62 strains, divided into 15 types of RAPD with the similarity of genetic patterns at >75–100%, and 3 unique strains isolated from mayonnaise salad, green salad, and juice. Cluster 2 consisted of 9 strains belonging to two types of RAPD and 1 unique strain from Chinese cabbage. The analysis of the degree of genetic diversity of psychrotolerant *B. cereus* strains with different toxin-forming potentials showed a large diversity of RAPD profiles in this group. The high degree of connection of *B. cereus* strains (>75–100%) from various environments, determined based on the Dice coefficient (3%), indicates a significant impact of secondary pollution on the qualitative environments. The different origins of *B. cereus* strains isolated from the same environment can also be evidenced by the lack of a relationship between the RAPD genetic patterns of the compared strains and their isolation environment / strain carrier.

DISCUSSION

The literature search confirmed a high discrepancy in prevalence values of *B. cereus* in foods. The analysis of minimally processed carrot conducted by Valero et al. (2002) demonstrated the presence of *B. cereus* in 42.8% of the analyzed samples whose contamination did not exceed 10^2 CFU/g, whereas in RTE vegetables studied by Kaneko et al. (1999), this prevalence was over twofold lower (20.1%). In our study, contamination of the analyzed food products did not exceed 10^3 CFU/g. Psychrotolerant *B. cereus* sensu lato was a large group of bacteria in most studied environments, i.e., fresh vegetable salads, fruit-vegetable juices, mayonnaise salads, and minimally processed vegetables. Reference materials confirm the presence of psychrotolerant *B. cereus* in various types of food products, including lasagne, béchamel sauce, thermally-treated basmati rice, carrot, cooked pasta (Samapundo et al., 2011), mayonnaise salads (Valero et al., 2007), or pasteurized vegetable puree (Choma et al., 2000). According to Carlin et al. (2010) and Francis et al. (1998), the differences in the sequences of *csp*A and 16S rDNA genes in psychrotrophic and mesophilic strains of *B. cereus* enable them to be used to differentiate strains with various minimal growth temperatures. The results obtained in our study lead to the conclusion that

the possession of a psychrotrophic gene 16S rDNA (p) and of a *cspA* gene is not a unique trait of the psychrotolerant strains. In the case of food products stored under chilled conditions, the prevalence of emetic strains of *B. weihenstephanensis*, which are psychrotrophic strains able to grow under such conditions, is significant. The percentage of strains able to grow under cold storage conditions used for food products (i.e., temperatures recommended by food producers) is not high and usually does not exceed 10% (Samapundo et al., 2011). Apart from the prevalence of *B. cereus* group bacteria and the level of food contamination with these bacteria, it is also significant that they have the potential to induce symptoms of intoxication, which, taken altogether, indicate the real health risk posed by their presence in food products. We focused our analyses on evaluating the toxigenic potential of psychrotolerant strains, which play a key role during cold storage. The obtained results confirm that genes encoding HBL and NHE toxins are relatively common representatives of the *B. cereus* group; however, the genes encoding components of the non-hemolytic toxin occur most frequently (Mendelsonn et al., 2004). Among the analyzed RTE food products, those in which analyses confirmed the presence of emetic *B. cereus* are noteworthy, i.e., samples of fresh vegetable salads, mayonnaise salads, and sushi. A common trait of most of these products was their vegetable ingredients, which were probably contaminated with *B. cereus* bacteria originating from the soil. Until recently, the prevalence of emetic *B. cereus* in the food environment has been regarded as sporadic, and their contribution in the group of *B. cereus* bacilli contaminating food has been estimated at ~3%, depending on the source (Apetroaie et al., 2005). According to some authors, the emetic strains of *B. cereus* may also sporadically synthesize enterotoxins, including mainly the NHE toxin (Ehling-Schulz et al., 2006). Our research results prove that emetic strains of *B. cereus* occur in food products much more frequently than expected, which might be associated with, among other things, the environment they had been isolated from. 23 of the 204 isolated strains belonging to the *B. cereus* group were potentially emetic (11.3%). The literature data indicate that the frequency of occurrence of the *cytK* genes in representatives of the *B. cereus* group is high and, according to Ngamwongsatit et al. (2008), accounts for 88.8% in *B. cereus* and for 83.9% in *B. thuringensis*. In our study, 29.8% of the isolated strains were able to produce this toxin. This difference might be associated with, among other things, the environment the strains were isolated from. It is believed that genes responsible for *cytK* synthesis, which occur more frequently in the population of diarrheal strains (Ehling-Schulz et al., 2006), have been acquired by these strains relatively recently as a result of their direct contact with enteric bacteria (Apetroaie et al., 2005). In *B. cereus* sensu lato, like in other bacteria, the process of recognition and response to a given stimulus requires an appropriate signal transduction mechanism to be activated, which allows the strain to adapt to environmental conditions (Carlin et al., 2010).

CONCLUSIONS

The results of this study indicate the presence and highly diverse toxicogenic potential of psychrotolerant strains of the *B. cereus* group. Therefore, the psychrotolerant pathogenicity of *B. cereus* sensu lato cannot be ignored. It is still being discussed whether the psychrotolerant strains used in this study, which are regarded as potentially toxigenic, produce toxins determined by a gene set of the strain at temperature of ≤10°C in an amount likely to induce symptoms of intoxication.

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