ECOTYPES OF *BACILLUS CEREUS* SENSU LATO

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Abstract

Bacillus cereus sensu lato is a group of bacteria common in the environment, which have an important impact on the activity of humans. Their taxonomy is still in doubt, mainly because of their properties encoded by plasmid-borne genes that may potentially undergo horizontal gene transfer. Here we present genetic and phenotypic data that support the idea of a bacterial group (named *B. cereus* sensu lato) instead of several independent taxa. We conclude that *B. cereus* sensu lato is composed of distinct, but closely related ecotypes.

Key words: cold-adaptation, 16S rRNA, sequencing, real-time RT-PCR, phylogeny

Introduction

Bacillus cereus sensu lato is a group of bacterial species that are common in the natural environment as well as in plant tissues, the intestines of animals and food matrices (Bartoszewicz and Czyżewska, 2017b). The special interest focused on these bacilli is associated with their properties. For instance, *B. cereus* and related species are able to biosynthesize several enterotoxins. Among them, two three-component enterotoxins (Nhe, HBL) and one cytotoxin (CytK) are involved in human food poisoning. On the other hand, rare isolates of *B. cereus* may also form a cereulide called an emetic toxin due to the list of symptoms caused after intoxication (Bartoszewicz et al., 2006). The next interesting property of *B. cereus* sensu lato is their ability to form spores. Apart from their resistance to heat or a lack of nutrients and water, they may also stand conditions that occur in food processing lines (high pressure, low concentration of oxygen, rapid temperature changes), which directly leads to contamination of ready-to-eat products, especially those made from milk and cereals, but not only. The next threat is linked to *Bacillus anthracis*, as this bacterium produces a complex toxin (composed of a protective antigen and lethal toxin or, in another variant, of protective antigen and edema factor), which is responsible for anthrax. Moreover, these bacilli also form a capsule that is not recognized by the host's immune system, so the infection course is usually rapid, and without proper treatment leads to death. Naturally, the mentioned bacteria also display positive properties. For instance, *B. thuringiensis* is used for the production of

bioinsecticides used worldwide in order to protect crops. Next, *B. toyonensis* shows some probiotic activities, so there is a hope to use it in the production of probiotics for cattle (Bartoszewicz and Czyżewska, 2017b).

Apart from numerous studies on the biology and ecology of *B. cereus* sensu lato, the taxonomy of this group is not coherent and it is still being discussed. It is suggested that due to high homogeneity in the 161S rRNA gene sequence (often >99% identity) and similarities in total DNA-DNA hybridization, all the rods pertaining to *B. cereus* sensu lato should be treated as one polymorphic species. However, there are some i portant phenotypic properties typical only for a small group of isolates. Only representatives of *B. thuringiensis* form crystal parasporal inclusions, which can be visualized by a specific staining technique or by the application of phase-contrast microscopy. In turn, *B. mycoides* forms colonies with rhizoidal morphology. Isolates of *B. anthracis* are non-motile (in opposition to the rest of the group), do not cause hemolysis on sheep blood agar, and are susceptible to penicillin. Finally, the still discussed *B. weihenstephanensis* differs in the sequence of the major cold-shock protein gene (*cspA*) and has a unique motif in its 16S rRNA gene. In the case of *B. weihenstephanensis*, mentioned features are associated with its psychrotolerant characteristics. What is more confusing, several discriminatory properties of particular taxa in the *B. cereus* group are encoded by genes located on large, potentially mobile plasmids and could be transferred during conjugation or lost during intensive proliferation in optimal conditions (Bartoszewicz and Czyżewska, 2017b). egarding these facts, the hypothesis of ecotypes instead of species in the *B. cereus* sensu lato has been proposed. Thus, we decided to evaluate the impact of the average temperature on the frequency of different ecotypes in samples from distinct climate zones. We also tried to compare ecological properties with data provided by nucleotide sequencing of the 16S rRNA of randomly selected isolates representing different *B. cereus* sensu lato taxa.

Materials and methods

Bacteria isolation. Thirteen different sampling sites in distinct locations were chosen in order to obtain samples. Each sample of soil (20 g) was aseptically collected using falcon tubes from a layer located approximately 2-5 cm below the ground surface after all the plants were removed. Next, soil samples were suspended (10% w/v) in LB broth, incubated for 10 minutes with vigorous shaking and subsequently pasteurized (72°C for 10 minutes) in order to eliminate competitive gram-negative microflora. Finally, 100 µl of the soil suspension was transferred onto the surface of MYP agar (mannitol-egg yolk-polymyxin agar, Oxoid), which is a selective medium for *B. cereus* sensu lato. After two days of incubation at 30°C (this temperature supports the growth of both psychrotolerant and mesophilic isolates), matt purple colonies with a visible zone of lecithin precipitation were selected and bacteria were transferred onto Columbia 5% Sheep-Blood agar (Oxoid) to investigate their hemolytic activities. Finally, after

purification by passages on LB agar using the streak plate technique, bacteria were frozen in LB with glycerol (v/v 1:1) at -80ºC.

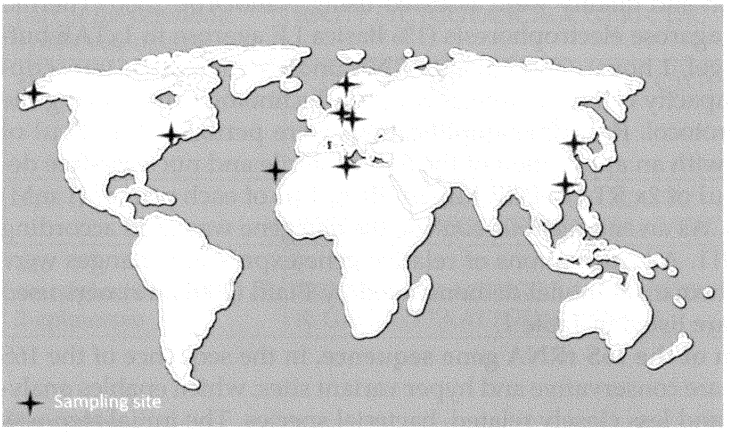

Figure 1. Approximate localization of sampling sites.

Identification of *B. cereus* sensu lato. Isolates were identified according to the procedure described elsewhere. Bacteria with rhizoidal colonies were identified as *B. mycoides/B. pseudomycoides* without further discrimination. Isolates with typical colonies were visualized under phase-contrast microscopy (Olympus BX-61). The presence of any parasporal inclusions (in case of sporulated cells incubated for 72 hours at 30ºC on T3 sporulating agar) was interpreted as confirming *B. thuringiensis*, while a lack of protein crystals suggested *B. cereus* or *B. mycoides*. Next, visible growth of isolate at 7ºC on LB agar after 9 days of incubation indicated psychrotolerance. Such isolates were tested for the presence of the specific sequence in the *cspA* gene (what is typical for *B. weihenstephanensis*). The lack of this genetic feature combined with adaptation for growth at cold conditions indicated psychrotolerant *B. cereus*. All isolates were also screened for the *pag*, *lef* and *cap* genes characteristic for *B. anthracis;* however, none of the samples gave a positive result.

Characterization of isolates. Bacteria were tested for their adaptation to different temperatures and growth rates, next their putative toxicity was assessed by the use of real-time RT-PCR technique (in relation to Nhe, HBL and CytK synthesis) or PCR (for investigation of cereulide synthetase gene complex). Psychrotolerance was assessed initially as described above; however, for growth rate at cold and optimal temperatures, liquid LB cultures were applied. In this case, chilled LB broth (5 ml in sterile glass tubes) was inoculated with 50 μl of a fresh culture of a particular strain (optical density OD_{600}=1,5). Immediately, the optical density of this sample was assessed by spectrophotometer (Jasko V 670) and incubation was started. Independent samples of each tested strain were incubated at 4ºC (10 days), 7ºC (10 days), 30ºC (2 days), 37ºC (2 days), 43ºC (2 days) and 54ºC (2 days). Next, every 24 hours, each sample density was measured to assess the proliferation dynamics. All cultures and measurements were repeated twice. In order to evaluate potential toxicity, total RNA was isolated

from randomly selected strains by the use of Total RNA Mini Plus Kit (A&A Biotechnology) according to the protocol provided by the manufacturer. Next, RNA concentration and quality were assessed using NanoDrop 2000 (Thermo Scientific) and by agarose electrophoresis (1% Basica LE agarose in 1xTAE buffer, 5V/cm of the gel, 1 hour). Good quality RNA underwent reverse transcription with High Capacity cDNA Synthesis Kit (Life Technologies) following the manufacturer's protocol. Real-time amplifications were performed in 20 μl of total volume and with an application of the 6 μl of sterile and nuclease-free deionized water, 10 μl of 2x RT HS-PCR Mix SYBR A, 1 μl of each primer (1 mM) and 2 μl of cDNA. As an endogenous control, the *udp* gene was used according to Reiter et al., 2011. All calculations of relative gene expression changes were based on the mathematical model demonstrated by Pfaffl (2001). Primers used for amplification are listed in Table 1.

Polymorphism of the 16S rRNA gene sequence. In the sequence of the 16S rRNA gene, there are conservative and hyper variant sites, which enables analyses of both, more and less closely related, bacterial species. The initial step was DNA isolation from overnight cultures using Blood and Tissue DNA Isolation Kit (Qiagen) and automatic station for nucleic acid isolation (QiaCube, Qiagen) with the protocol for gram-positive bacteria. Obtained DN was tested using NanoDrop 2000 and then was used as a template for amplification as described elsewhere (Bartoszewicz et al. 2009). Amplified samples were purified using Clean-up kit in order to remove any residual nucleotides, primers, and enzymes, according to the protocol provided with the kit. Next, samples were amplified in GeneAmp 9700 thermocycler (Applied Biosystems) using BigDye Terminator v4.1. kit (Life Technologies), and residual terminators were removed by the use of ExTerminator Kit (A&A Biotechnology) with the typical procedure recommended by the manufacturer. Finally, samples were sequenced in DNA sequencer ABI 3150 (Life Technologies). Obtained sequences were visually examined in Chromas Lite software and imported to BioEdit software for performing sequence alignment. In the next step, sequences were exported to Mega 6 software. Before the reconstruction of *B. cereus* sensu lato ph logeny, we performed tests for the optimal evolutionary model. On the basis of the BIC scores, the maximal likelihood method with general time reversible model (GTR+G+I) was applied to construct a dendrogram.

Table 1. Sequences of primers used in real-time PCR and PCR assessment of *B. cereus* sensu lato.

Gene	Sequence 5'-> 3'	Reference
nheA part of the Nhe operon	F: TTTAATTGCGGGGTTATTGG R: ACTACTCATCGCGCTCACC	Melnick et al. (2012)
hblA part of the HBL operon	F: CCTTGCAAAAGGCTGGATTA R: TCGTGTCCCAAGTAACAGC	Melnick et al. (2012)
cytK	F: GATAATATGACAATGTCTTTAAA R: GGAGAGAAACCGCTATTTGT	Świecicka et al. (2006)

Gene	Sequence 5'-> 3'	Reference
cesA part of the cereulide synthetase operon	F: GGTGACACATTATCATATAAGGTG R: GTTTTCTGGTAACAGCGTTTCTAC	Ehling-Schulz et al. (2005)
16S rRNA	F: CGCGGGATCCGAGTTTGATCCTG-GCT R: GGCCGTCGACACGG(A/C) TACCTTGTTACGACT	Lechner et al. (1998)
cspA	F: CGAATTTGATAATGTGTGGATTC R: CCCGGATCCGGTTACGTTA(G/C)C	Lechner et al. (1998)
udp acetylglucosamine 2-epimerase	F: ACTAGAGAAACTTGGAAATGATCG R: GACGCTTAATTGCACGGAAC	Reiter et al. (2011)

Results

During the entire study, 400 *B. cereus* sensu lato isolates were obtained from soil samples collected during two years in China (2 sampling sites, N=84 isolates), Poland (3 sampling sites, N=71), Germany (1 sampling site, N=59), Egypt (2 sampling sites, N=66), Madera (2 sampling sites, N=56), USA (Alaska, 1 sampling site, N=15) Sweden (1 sampling site, N=20), and finally Rhode Island (1 sampling site, N=39).

The distribution of particular taxa pertaining to the *B. cereus* group is presented in Figure 2.

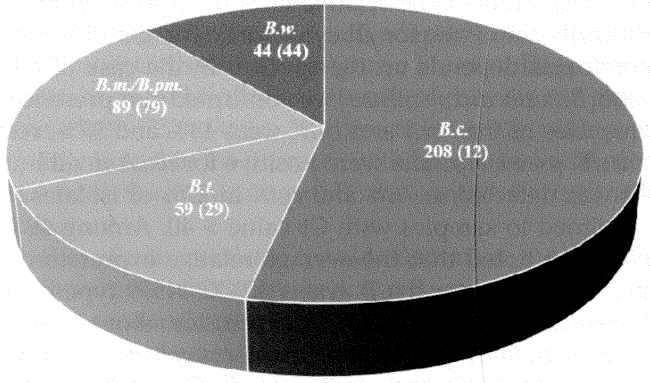

Figure 2. Distribution of *B. cereus (B.c.), B. thuringiensis (B.t.), B. mycpides/B. pseudomycoides (B.m./B.pm.)* and *B. weihenstephanensis (B.w.)* in soil samples. Presented values indicate total number of isolates and (in brackets) number of cold-adapted isolates.

As shown, the dominant species in all samples was *B. cereus* representing more than half of the isolates. However, only 12 of them (5.7%) shared cold-adapted

characteristics but did not possess the *cspA* gene or specific motif in the 16S rRNA gene characteristic to *B. weihenstephanensis*. The frequencies of remaining species were quite similar, but psychrotolerance was more common in *B. weihenstephanensis* (all isolates) and *B. mycoides/B. pseudomycoides* (89%), while surprisingly half of the *B. thuringiensis* isolates proliferated at 7ºC and in most cases also possessed the *cspA* gene detected in the PCR screening. Apart from the fact that the presence of each species in samples from different climate zones were similar, samples were significantly different in the percentage of psychrotolerant strains. In the Alaskan soil, as well as in samples from Sweden, we noted 55% of all psychrotolerant *B. thuringiensis* isolates. Moreover, samples contained significantly more *B. mycoides/B. pseudomycoides* and *B. weihenstephanensis* than samples from the remaining locations. On the other hand, samples obtained from Egypt, Madera and one of the Chinese sampling sites (Shenzhen) were devoid of psychrotolerant representatives of *B. cereus* sensu lato. Finally, soil obtained from Rhode Island (USA), Poland, Germany and China (approx. 70 km from Pekin) showed a moderate frequency of cold-adapted bacilli. For instance, both samples from Poland contained totally 39 *B. cereus* isolates (among them 3 were psychrotolerant), 5 psychrotolerant *B. thuringiensis* isolates (as well as 9 mesophilic ones), 7 *B. weihenstephanensis* and 11 *B. mycoides/B. pseudomycoides* (all psychrotolerant). Similarly, in soil from Germany, we found 31 *B. cereus* (1 psychrotolerant), 11 *B. thuringiensis* (including 3 psychrotolerant ones), 12 *B. mycoides/B. pseudomycoides* (among them 11 were cold-adapted) and finally 5 *B. weihenstephanensis*.

The toxicity of *B. cereus* sensu lato was assessed using real-time RT-PCR in the case of 50 randomly chosen strains representing each species proportionally. As reference strains, we used the *B. cereus* ATCC 14579 type strain (for *nheA*, *hblA*, and *cytK*). The value of the relative level of expression for the reference strain was arbitrarily set to one for all cytotoxins. The distribution of the genes encoding enterotoxins depends on the species. In the case of all *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* we confirmed the presence of the *nheA* gene; the frequencies of the *hblA* and *cytK* were 48% and 37%, respectively. In turn, *B. mycoides/B. pseudomycoides* were positive for *nheA* in all but one isolate. However, *hblA* was detected in 76% and *cytK* in 22% of isolates. The negative results were assigned to samples with Ct value > 40. Among 49 isolates positive for the *nheA*, we noted that the average relative expression rate was 0.45 of the reference expression for the *B. cereus* ATCC 14579 type strain. However, *B. mycoides/B. pseudomycoides* exhibited an expression about 25% lower in comparison to *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis*. In turn, the *hblA* gene was expressed more efficiently in *B. mycoides/B. pseudomycoides* (its average rate was 1.54 times higher than in the case of reference *B. cereus* ATCC 14579 strain) and the remaining taxa showed expression rates ranging 0.47 (*B. thuringiensis*) to 0.67 (*B. cereus*). Finally, the *cytK* gene was expressed efficiently only in three *B. cereus* isolates (average expression rate 1.13 of the reference expression), while in the case of *B. thuringiensis* we noted only 0.44 of the reference, and in *B. mycoides/B. pseudomycoides* or in *B. weihenstephanensis* it did not exceed 0.32. None of the strains was positive in the case of the *cesA* gene necessary for the biosynthesis of cereulide.

Tests of growth proved that *B. cereus* sensu lato can multiply at a wide range of temperatures. *B. mycoides/B. pseudomycoides* were able to grow at 4ºC (33% of isolates) and at 7ºC 89% were multiplicated on solid and liquid media. Next, 50% of *B. weihenstephanensis* grew well at 4ºC and all isolates were proliferating at 7ºC. However, none of *B. mycoides/B. pseudomycoides* and *B. weihenstephanensis* grew at or above 43ºC. Among *B. thuringiensis* one psychrotolerant isolate grew at 4ºC, while the rest of the cold-adapted strains multiplicated at 7ºC and at 30-37ºC, but not at higher temperatures. *B. cereus* isolates were not able to grow at 4ºC, but almost 6% were proliferating at 7ºC. Those psychrotolerant isolates were also able to multiply at 30ºC, 37ºC, and 43ºC, but not at 54ºC. In general, no isolates were growing at 54ºC (the temperature, which is a diagnostic feature for thermotolerant *B. cytotoxicus*).

The polymorphism of the isolates was assessed with the aid of 16S rRNA gene sequencing from 147 randomly selected isolates. Obtained data were used for the phylogeny reconstruction of the isolates in the context of their origin, taxonomy, toxicity, and temperatures of growth. A dendrogram depicting relationships between isolates belonging to the *B. cereus* sensu lato group is presented in Figure 3. On the basis of all presented data we can conclude that *B. cereus* and related taxa exhibited a high level of genetic polymorphism in the 16S rRNA gene sequence. However, the number of variable sites suggests that variant fragments of this gene constitute up to 1% of the entire examined fragment (about 1420 kbp). Thus, all the members of the *B. cereus* sensu lato have their 16S rRNA gene sequence with an identity above 99%. The next important outcome of the study is linked to the group signed as 'cold-adapted' bacilli. Among them are members of *B. weihenstephanensis*, *B. mycoides/B. pseudomycoides*, but also representatives of *B. thuringiensis* and *B. cereus.* Interestingly, apart from typically psychrotolerant *B. mycoides/B. pseudomycoides* or *B. weihenstephanensis*, cold-adapted strains comprised also both *cspA*-positive *B. thuringiensis* and *cspA*-negative *B. cereus.* Deeper analysis showed that psychrotolerant bacteria constitute two branches on the phylogenetic tree. On the first one (clade C2 in Figure 3), there were strains with psychrotolerance that could be easily linked to the presence of the *cspA* gene, while the second one, named clade C1 (with the domination of *B. cereus* and *B. thuringiensis*), was devoid of this genetic feature.

Apart from those findings related to cold-adaptation, the 16S rRNA gene does not allow for differentiation between particular *B. cereus* sensu lato. In some cases, isolates of *B. cereus* are much more similar to *B. thuringiensis* than to other representatives of *B. cereus.* For example, strains pertaining to clade A were representing *B. cereus* and *B. thuringiensis.* However, we did not find any case where the identical nucleotide sequence of the 16S rRNA gene could be observed for more than one species. Clonal *B. cereus* sensu lato (the biggest group consisted of seven *B. mycoides/B. pseudomycoides* isolates from clade C2 and a few other groups composed of four isolates identified as *B. cereus* from clade D), *B. weihenstephanensis* (from clade C2) or *B. mycoides* (also from C2), respectively were tested to assess if they share the same phenotypic characteristics. According to the results, the range of growth temperatures was the same and the ratios of expressions of the *nheA*, *hblA* and *cytK* were not different significantly within the group of species with

an identical 16S rRNA gene sequence. What could be added (data not shown), clonal isolates were also identical when examined using REP-PCR fingerprinting technique. Surprisingly, we did not observe any correlation between the sequence of the 16S rRNA and the origin of the strains. For example, the mentioned clonal group of seven identical *B. mycoides/B. pseudomycoides* isolates was composed of isolates from Poland, both Chinese sampling sites, and from Rhode Island (USA).

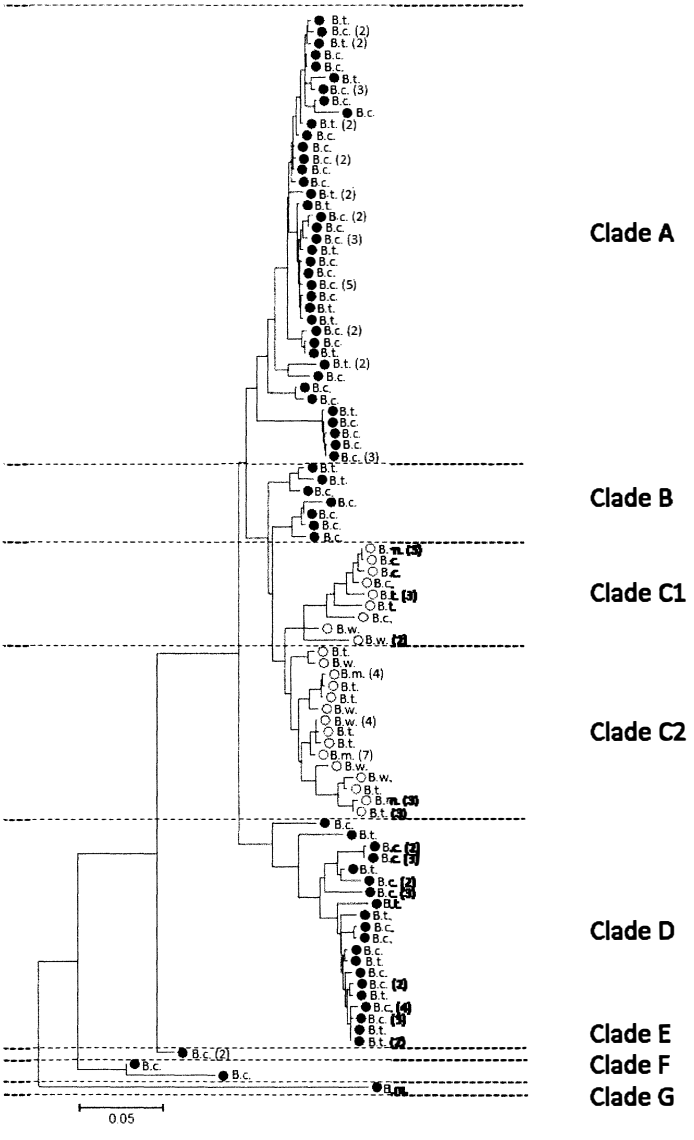

Figure 3. Dendrogram depicting phylogenetic relationships between selected isolates of *B. cereus* sensu lato. Phylogeny reconstruction using minimal evolution method and general time reversible model (GTR+G+I)

using Mega 6.0 computer software. Cold-adapted bacilli are marked with an empty circle, while mesophilic isolates are marked with black wheels. Species affiliation is as follows: B.c. – *B. cereus*, B.t. – *B. thuringiensis*, B.w. – *B. weihenstephanensis*, B.m. – *B. mycoides/B. pseudomycoides*. Numbers in brackets display the number of isolates with identical 16S rRNA gene sequence. Scale at the bottom corresponds to the genetic distance.

Discussion

B. cereus sensu lato is an interesting object for intensive study on the border of ecology, medicine, veterinary science and industry (especially the dairy industry). Their huge impact on human activities and the health status of people and animals cannot be omitted. However, still there are some important gaps in the general knowledge about these bacteria. Their taxonomy is doubtful. Some evidence supports the hypothesis of one polymorphic species while other evidence suggests that we are dealing with different taxa (Bartoszewicz and Czyżewska, 2017). Recently, a few authors, basing on different and independent results have suggested that we should change our perception of *B. cereus* sensu lato. The approach that leads to the recognition of distinct species has practical advantages (eg in medical diagnostics), but it is not sufficiently supported by experimental data. In turn, treating all diverse *B. cereus* sensu lato as one species with several unique properties characteristic to only one taxon is also wrong. Thus, we should rather treat *B. cereus* sensu lato as a bacterial group (with a rank between genus and species) and focus our attention on the properties of particular, interesting strains.

The percentage of psychrotolerant and mesophilic *B. cereus* sensu lato varies between samples; however, we see a strong correlation between climate at the sampling site and the range of temperatures of growth. In previous studies, the highest rate of cold-adapted bacilli was noted in the temperate climate zone (Bartoszewicz et al., 2009), because it could give an advantage in competition with soil microflora at these conditions. For example, the average temperature in Poland (on the basis of data from 1981-2010) is 7.3ºC with the hottest month (July) with 18.0ºC and the coldest one (January) with -3.1ºC. For comparison, in Cairo (Egypt) the coldest month is also January with 14.0ºC, while the highest temperatures are observed in July (28.4ºC), so these conditions much better fit to mesophiles. Interesting questions are often asked about the development of cold-adaptation. This feature, initially addressed only to *B. weihenstephanensis* (Lechner et al., 1998) and *B. mycoides* (Jensen et al., 2003) appears to be more common. Bartoszewicz et al. (2009) proved the existence of cold-adapted *B. thuringiensis* and suggested that they appeared as a result of a conjugation event between *B. weihenstephanensis* (as a recipient) and *B. thuringiensis* (as a plasmid with *cry* genes donor). However, MLST studies (Bartoszewicz and Marjańska, 2017; Bartoszewicz and Czyżewska, 2017) showed another subgroup among

B. cereus sensu lato with a psychrotolerant phenotype. In combination with current results (the lack of the *cspA* gene in some of the psychrotolerants) it may indicate the independent origin of cold-adaptation and possible different pathways for achieving this feature.

The lack of any correlation between climate and toxicity could also be explained. The ability of toxin biosynthesis is useful in different conditions. *B. thuringiensis* uses its insecticidal toxins to break the intestinal barrier of larvae, which results in a leak of the hemolymph into the intestine and provides nutrients for bacteria (Bartoszewicz and Czyżewska, 2017). On the other hand, food-poisoning toxins that were examined in the present study may have a quite different practical application for bacteria. One of the theories assumes that those toxins are used for quorum sensing by the bacteria (Raymond et al., 2013). In this context, their synthesis may be favored in habitats with high concentrations of *B. cereus* sensu lato, for example in food matrices and in the dairy industry, where biofilms containing *B. cereus* and related species could be formed.

Conclusion

Summing up all the mentioned findings, we should note that *B. cereus* sensu lato is rather one polymorphic species with several distinct ecotypes. Natural selection for the properties which lead to increased fitness in specific conditions is the force which forms closely related clades more typical to occupied habitats than to taxonomic affiliations. Moreover, horizontal gene transfer may complicate the taxonomy of *B. cereus* sensu lato leading to the acquisition of properties typical to the different taxa, as was suggested for *B. weihenstephanensis* with features of *B. thuringiensis*. Finally, climate conditions appear to be the most powerful force for the selection of psychrotolerant members of *B. cereus* sensu lato; however, cold-adaptation is probably characteristic to two different evolutionary lineages of the entire group.

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