Toxin Genes of Bacillus cereus

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Bacillus cereus is a ubiquitous soil bacterium responsible for two types of food-associated gastrointestinal diseases. While the emetic syndrome is caused by the cyclic depsipeptide cereulide, proteinaceous enterotoxins provoke the diarrheal disease. Here, an overview on the distribution of the main toxin genes/operons *ces* (encoding cereulide), *hbl* (encoding the tripartite hemolysin BL), *nhe* (encoding the tripartite non-hemolytic enterotoxin), and *cytK* (encoding the single protein cytotoxin K) within the *B. cereus* group is given.

Keywords: Bacillus cereus ; hemolysin BL ; non-hemolytic enterotoxin ; cytotoxin K ; cereulide ; pore formation ; cytotoxicity ; food poisoning

1. Introduction

Bacillus cereus is estimated to be responsible for 1.4%–12% of all food poisoning outbreaks worldwide[1]. In the European Union, bacterial toxins (*Clostridium, Staphylococcus* and *B. cereus*) accounted for 17.7% (2016) and 15.9% (2017) of all registered food- and water-borne outbreaks, which ranked them second behind *Salmonella*[2][3]. With 98 registered outbreaks in the EU in 2018, *B. cereus* toxins ranked in fifth place behind *Salmonella, Campylobacter*, the norovirus and *Staphylococcus* toxins. Among these was also one large food poisoning outbreak with more than 100 affected persons. Furthermore, six fatal cases were attributed to bacterial toxins (*Clostridium botulinum, Clostridium perfringens* and *B. cereus*) [4].

Basically, *B. cereus* is responsible for two types of gastrointestinal diseases. The emetic kind of illness is mainly characterized by nausea and emesis, which appear as soon as half an hour after consumption of the contaminated food and are clinically indistinguishable from intoxications with *Staphylococcus aureus* enterotoxins[5]. In this classical food intoxication, the emetic toxin cereulide is pre-formed during vegetative growth of *B. cereus* in foodstuffs and the consumption of the bacteria is not necessary [6]. Indeed, there are several reports of outbreaks where only the cereulide toxin was detected in the food, but no bacteria could be isolated[7]. Nevertheless, it is generally thought that at least 10^3–10^5 *B. cereus* per g food are needed to produce cereulide in disease-provoking concentrations [5][6][7][8][9]. Cereulide is a cyclic dodecadepsipeptide with a molecular weight of 1.2 kDa. The basic repeated amino acid sequence [D-*O*-Leu D-Ala L-*O*-Val D-Val]$_3$ is extremely stable towards heat, acid or digestive enzymes and, thus, the toxin can hardly be removed or inactivated[10][11][12]. Usually, the emetic form of disease is self-limiting and symptoms disappear after 6–24 h. Nevertheless, some severe and fatal outbreaks mostly related to liver failure are reported [10][13][14][15][16][17][18][19][20][21][22][23]. Due to the ubiquitous nature of the pathogen and its production of highly resistant spores, *B. cereus* is frequently found in various kinds of food[24][25][26]. Historically, starchy foodstuffs such as rice or pasta are connected to food intoxications with emetic *B. cereus*, but more recently evidence is growing that emetic *B. cereus* are much more volatile than once thought. The comprehensive analysis of a total of 3654 food samples obtained from suspected food-borne illnesses with a preliminary report of vomiting, collected over a period of seven years, revealed that emetic *B. cereus* strains were detected in a broad diversity of foods, including vegetables, fruit products, sauces, soups, and salads as well as milk and meat products[7].

The second, diarrheal form of food poisoning is also associated with a variety of different foodstuffs[27]. This form of disease manifests mainly in diarrhea and abdominal cramps, similar to food poisoning by *Clostridium perfringens* type A[5]. Symptoms occur after approximately 8–16 h. This incubation time is typical for toxico-infections, in which the toxins are produced by viable bacteria inside the human intestine[5][28][29]. Unlike cereulide, enterotoxins pre-formed in foods most likely do not contribute to the disease, as they are considered sensitive towards heat, acids or proteases. Thus, vegetative *B. cereus* and, especially, spores must be consumed. The infective dose is estimated between 10^5–10^8 cfu/g[11][30] or 10^4–10^9 cfu/g[9][29] vegetative cells or spores. The course of disease is mainly mild and—after approximately 12–24 h—self-limiting. Fatal outbreaks are only very rarely reported[31]. A food infection with enteropathogenic *B. cereus* can be seen as a multifactorial process, as a number of individual steps have to be considered before the onset of the disease, including prevalence and survival of *B. cereus* in different foodstuffs, survival of the stomach passage, germination of spores, active movement towards and adhesion to the intestinal epithelium, enterotoxin production under intestinal conditions, as well as the influence of consumed foods and the intestinal microbiota on these processes.

2. Distribution of Toxin Genes

2.1. Prevalence among Isolates from Environment, Foods and Outbreaks

B. cereus is a ubiquitous soil bacterium and can thus be found worldwide in the ground, in dust, or on different foods. Early studies pointed to an occurrence of diarrheal or emetic outbreaks according to country-specific dietary habits, with the emetic form manifesting in Great Britain or Japan, and the diarrheal form rather in Northern Europe or the USA[32][33]. Lately, both syndromes have been reported from all over the world. Basically, emetic strains are found less frequently in foods as well as in the environment than enteropathogenic strains[27][34][35]. In a multitude of studies, new isolates were screened for the presence of the toxin genes *nhe (ABC), hbl (CDAB), cytK (1,2), entFM*, and *ces*. In some studies, the presence of *bceT* (enterotoxin T) was also assessed; however, its enterotoxic capacity is disproven[36][37][38]. Virulence/enterotoxin gene patterns are compiled for *B. cereus* which has been mainly isolated from foods, but also from clinical, soil and environmental samples worldwide. Generally, those patterns are highly diverse[39][40][41][42][43][44][45].

Common distribution of the toxin genes is approximately 85%–100% *nhe (ABC)*, approximately 40%–70% *hbl (CDA)*, approximately 40%–70% *cytK-2*, very few *ces*+, typically no *cytK-1*+, and—if tested—approximately 60%–100% *entFM*, which has been detected in studies from Europe [42][46][47][48][49][50][51][52]], South America[53][54], North America[39][55], Asia[56][57][58][59][60][61][62][63]and Africa[64][65][66]. Nevertheless, in some studies, a connection was established between toxin gene patterns and geographical location of the isolates. Drewnowska et al. found that strains possessing *nheA, hblA* and *cytK-2* were predominant in regions with arid hot climate, and were comparably rare in continental cold climates[67]. This is supported by other studies suggesting that geographic origin might have an impact on the conservation of *hblA* among *B. cereus* populations[68][69][70]. Zhang et al. also claim a “regional feature for toxin gene distribution”[71].

Besides geographical location, toxin gene patterns seem to be also influenced by the kind of foodstuffs analyzed. For instance, Berthold-Pluta et al. found higher prevalence of *nhe*+ and *hbl*+, but lower prevalence of *ces*+ strains in food products of animal than of plant origin[72]. Rossi et al. showed that strains from dairy products had significantly lower *cytK-2* and *hblCDA* prevalence than strains from equipment or raw milk[73], and Hwang and Park found *hbl* in >95% of tested ready-to-eat (RTE) foods, but only in 30% of infant formulas. Furthermore, the prevalence of *cytK-2* was comparably low in the latter food[74].

Studies were also conducted comparing food related and food poisoning related strains. Santos et al. showed that food poisoning strains had a higher occurrence and higher genetic diversity of *plcR-papR, nheA, cytK-2, plcA*, and *gyrB* genes than strains isolated from soil or foods[75]. *CytK* and the combination *hbl-nhe-cytK* were more often found among food poisoning related than among food related strains[49][50][76]

Generally, all *B. cereus* isolates can be categorized into seven different toxin profiles: A (*nhe*+, *hbl*+, *cytK*+), B (*nhe*+, *cytK*+, *ces*+), C (*nhe*+, *hbl*+), D (*nhe*+, *cytK*+), E (*nhe*+, *ces*+), F (*nhe*+), and G (*cytK*+)[46]. In fact, the *hbl* genes alone or a combination of *ces* and *hbl* have only been reported for the very few emetic *Bacillus weihenstephanensis* isolates described so far[77]. There are further studies showing “unusual” results, particularly low or no prevalence of *nhe*[43][72][78][79][80][81][82] or extraordinarily high prevalence of *hbl*[74][83][84][85][86] or *ces*[87], which must be interpreted cautiously, especially as *nhe* is well known for its molecular heterogeneity[46][49][50]. Thus, the choice of detection methods, especially primer pairs for *nhe*, can have a crucial influence on the results.

However, it has to be mentioned that the presence of enterotoxin genes or a certain toxin gene profile does not necessarily allow conclusions on the toxic activity of a *B. cereus* isolate[51][88]. In our own studies, we chose pairs of strains with an identical toxin gene profile, but one strain exhibited high and the other low toxic activity both under routine laboratory and simulated intestinal growth conditions[89][90]. The reasons for this are so far not completely understood, but it is believed that highly variable and strain-specific mechanisms in toxin gene transcription, posttranscriptional and posttranslational modification and protein secretion are involved.

2.2. Presence within the B. cereus Group

In many of the studies mentioned in Section 2.1, often only *B. cereus sensu lato (s. l.)* strains are investigated, meaning there is no differentiation between the members of the *B. cereus* group. In routine microbiological diagnostics, only “presumptive” *B. cereus* are detected on selective culture media according to international standards (ISO 7932:2005-03)

[91][92]. The *B. cereus* group comprises at least eight species: *B. anthracis, B. cereus sensu stricto (s. s.)*, *B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. cytotoxicus* and *B. toyonensis*[93][94][95][96]. Additionally, more and more species such as *B. wiedmannii, B. bingmayongensis, B. gaemokensis, B. manliponensis*, and others are described[97][98][99][100][101]. Generally, they exhibit high genetic similarities and, thus, it has been suggested that they be considered as one species[5][102][103] or to completely change the taxonomic nomenclature of the *B. cereus* group[104]. Species definition is historically based on phenotypes or clinical and economical relevance. While the unique characteristics of *B. anthracis*, emetic *B. cereus* and *B. thuringiensis* are located on plasmids[103], the enterotoxins are chromosome-coded and can thus be present throughout the *B. cereus* group. This is particularly problematic for the assessment of *B. thuringiensis*, which is frequently used as biopesticide worldwide[105][106][107]. *B. thuringiensis* has been isolated from a variety of foodstuffs and the presence of the enterotoxin genes *nhe, hbl* and *cytK-2* has been shown, with similar percentages as for *B. cereus*[55][58][70][88][108][109][110][111][112][113][114][115][116][117][118][119][120][121][122][123], while *ces* genes have not been found[124][125]. Enterotoxin production and cytotoxic activity have also been shown [55][111][113][114][115][121][126][127][128][129], and *B. thuringiensis* could therefore be involved in food poisoning outbreaks[130]. Consequently, it was debated whether the *B. thuringiensis*-associated biopesticides represent a risk for public health. To clarify this question, there is a demand for simple methods enabling a clear discrimination between *B. cereus* and *B. thuringiensis* in routine food and clinical diagnostics as well as for unequivocal identification of the strains used as biopesticides[124].

Next to *B. cereus* and *B. thuringiensis*, further species of the *B. cereus* group were isolated from foods and the presence of enterotoxin genes was proven, such as *B. anthracis*[46], *B. mycoides*[40][41][46][68][69][131][132], *B. pseudomycoides*[40][69], *B. toyonensis* [135], and *B. weihenstephanensis* [40][46][69][133][134]. It has also been shown that *Bacillus* spp. outside the *B. cereus* group can harbor one or more enterotoxin genes [135][136]. For instance, Mäntynen and Lindström found *hblA*+ *B. pasteurii* DSM 33, *B. smithii* DSM 459, and *Bacillus* sp. DSM 466 [68]. *Nhe* and/or *hbl* genes were also detected in *B. amyloliquefaciens, B. circulans, B. lentimorbis*, and *B. pasteurii* [137]. On the other hand, From et al. found no enterotoxin genes outside the *B. cereus* group in the strains analyzed [138].

According to MLST (multi-locus sequence typing), AFLP (amplified fragment length polymorphism) and whole genome sequencing, the *B. cereus* group was first assigned to three phylogenetic groups (clades)[139], then seven (*panC* types) [94], and later nine[118], which do not correlate with species definition[103]. Prevalence of enterotoxin genes and their profiles were also compared to phylogenetic groups. *B. cereus* isolates from dairy products in Brazil with approximately 50% *cytK-2* and *hbl*, and approximately 85% *nhe* were mostly assigned to phylogenetic group III. Group IV and V showed significantly higher prevalence of *hblCDA* and group IV showed additionally higher prevalence of *cytK-2* [73][94]. In another study on dairy isolates, strains of clade IIIc had no *hblCDA* operon, while strains of clade IV carried it and produced the Hbl toxin, whereas strains of clade VI carried the gene but did not produce the toxin [118]. Furthermore, a broad distribution of enterotoxin genes among seven phylogenetic clades, in which dairy-associated isolates were divided, was shown [88]. Okutani et al. investigated the genomes of 44 *B. cereus* group isolates from soil, animal and food poisoning cases in Japan. Strains were assigned to four different *panC* types and five different clades. The *nhe* operon was found in all strains tested, while *ces* was detected only in the food poisoning strains. When the presence or absence of virulence-associated genes was statistically analyzed, the majority of soil and animal isolates was part of overlapping clusters, while three of the four food poisoning isolates formed a distinct cluster [140]. Furthermore, the *hbl* and the *ces* genes were significantly correlated with the phylogenetic group[140][141]. Several further studies suggested that the toxic potential of *B. cereus s. l.* strains depends rather on the phylogenetic group than on the species[94][118][142].

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