

The cytotoxic potential of *Bacillus cereus* strains of various origins

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ARTICLE INFO

Keywords:

Bacillus cereus
Molecular characterization
Cytotoxicity
Genetic signature

ABSTRACT

B. cereus is a human pathogen associated with food poisoning leading to gastrointestinal disorders, as well as local and severe systemic infections. The pathogenic spectrum of *B. cereus* ranges from strains used as probiotics in humans to lethal highly toxic strains. In this study, we gathered a collection of 100 strains representative of the pathological diversity of *B. cereus* in humans, and characterized these strains for their cytotoxic potential towards human cells. We analyzed the correlation between cytotoxicity to epithelial and macrophage cells and the combination of 10 genes suspected to play a role during *B. cereus* virulence. We highlight genetic differences among isolates and studied correlations between genetic signature, cytotoxicity and strain pathological status. We hope that our findings will improve our understanding of the pathogenicity of *B. cereus*, thereby making it possible to improve both clinical diagnosis and food safety.

1. Introduction

B. cereus sensu stricto belongs to a larger group of bacteria commonly named the « *Bacillus cereus* group ». The group is constituted of eight species: *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. anthracis*, *B. thuringiensis*, *B. cereus sensu stricto* (Bc), *B. cytotoxicus* and *B. toyonensis*. Differentiation of species within the *B. cereus* group is complex due to the genetic proximity between the members of the group (Liu et al., 2015). Originally, species in the group were classified on the basis of phenotypic differences, distinct virulence trait and the presence of extrachromosomal elements that reflect the specie's virulence spectrum. Currently, the most widespread classification system of the *B. cereus* group is based on the sequencing of *panC* housekeeping gene, which encodes for the pantoate-beta alanine ligase C. Using this classification, seven phylogenetic groups have been determined based principally on their range of growth temperature (Guinebretière et al., 2002). *B. cereus* can be found in several group among which the groups III and VII, which comprise species associated with high toxicity (Guinebretière et al., 2010). However, a proper differentiation between the group members is still difficult and the pathogenic potential of the Bc strains is broad and diverse.

Bacillus cereus group species are sporulating ubiquitous bacteria that form biofilms. These properties allow them to withstand most cleaning and decontamination processes (Ramarao et al., 2015). It is not

surprising to find them at all food processing steps and in different hospital environments. Bc is the second causative agent of confirmed and suspected foodborne illness in France and the third in Europe (Journal, 2009). Bc is found in many raw and processed foods. The increasing use of refrigerated foods and the related increase in the number of collective cases of food-borne poisoning involving Bc raise questions about how far-reaching the danger actually is and how it could be controlled. Bc causes two types of food-borne illnesses. The emetic form is caused by the ingestion of cereulide, a peptide produced by the bacterium and already present in the ingested food (Ehling-Schulz et al., 2004). The diarrheal form is generally associated with the ingestion of bacteria producing toxins (ie: Nhe, Hbl, CytK) (Stenfors Arnesen et al., 2008). Symptoms usually last less than 24 h (Decousser et al., 2013) but several fatal cases of bloody diarrhea and emetic poisoning have been reported (Kotiranta et al., 2000; Lund et al., 2000; Naranjo et al., 2011).

Bc is also associated with severe local and systemic human infections, posing a public health problem (Bottone et al., 2010). The increasing frequency with which such non-gastrointestinal diseases are being reported highlights the importance to study this emerging pathogen (Bottone et al., 2010). In particular, Bc induces systemic infections, especially in premature newborns, leading to the patient death in about 10% of cases (Gaur et al., 2001; Glasset et al., 2018; Lotte et al., 2017; Ramarao et al., 2014; Veysseyre et al., 2015).

Bc possess several toxin genes, which play a role during Bc virulence.

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Table 1Genetic signatures (GS) of *B. cereus* strains according to gene detection and repartition in the FBO (F), Clinical (C) and non-pathogenic (NP) strain collections.

Genetic signature	Number of strains	Genes detected						Strain collections			
		<i>cytK-1</i>	<i>cytK-2</i>	<i>ces</i>	<i>hlyII</i>	<i>nheABC</i>	<i>hblCDA</i>	% total	F (n = 39) In %	C (n = 41) In %	NP (n = 20) In %
GS1	34	-	-	-	-	+	-	21,0	14,3	33,9	-
GS2	28	-	+	-	-	+	+	26,9	38,1	21,4	19,0
GS3	25	-	-	+	-	+	-	13,4	19,0	14,3	-
GS4	18	-	+	-	-	+	-	10,1	4,8	16,1	4,8
GS5	18	-	-	-	+	+	+	3,4	2,4	3,6	4,8
GS6	10	-	+	-	+	+	+	3,4	4,8	3,6	-
GS7	8	+	/	/	/	/	/	3,4	9,5	-	-
GS8	6	-	-	-	-	BC	AD	9,2	-	-	52,4
GS9	4	-	+	-	+	+	-	2,5	4,8	1,8	-
GS10	5	-	-	-	-	+	+	3,4	-	1,8	14,3
GS11	1	-	+	+	-	+	-	0,8	-	1,8	-
GS12	2	-	-	-	+	+	-	2,5	2,4	1,8	4,8

/: primers used are unable to detect the genes in GS7 group except CytK-1.

BC: only *nheB* and *nheC* detected by PCR designed.AD: only *hblA* and *hblD* detected by PCR designed.

Nhe complex is composed of three different proteins, A, B and C that are encoded by the *nheA*, *nheB* and *nheC* genes, respectively (Jebberger et al., 2015). Nhe is recognized as the major diarrheal toxin of *B. cereus*. Hbl is composed of a binding protein B encoded by the *hblA* gene and two lytic components L1 and L2 encoded by *hblC* and *hblD* genes, respectively (Beecher and Wong, 1994). The secreted enterotoxins Hbl and Nhe are more abundant in clinical and food poisoning strains than in environmental strains (Guinebretière et al., 2002), and a correlation has been shown between cytotoxicity and concentration of Nhe in *Bc* supernatant (Moravek et al., 2006). These toxins provide an indication of the strain toxicity potential but are not, alone, sufficient to discriminate hazardous from harmless strains (Glasset et al., 2018; Guinebretière et al., 2002; Martinez Blanch et al., 2009; Ramarao et al., 2020).

There are two variants of CytK that share 89% of identity: CytK1, and CytK2. CytK1 was identified from a strain that contributed to the death of three people (Lund et al., 2000). CytK2 is five times less toxic than CytK1, but it seems more frequently associated with strains causing Food Borne Outbreaks (FBO) (Ramarao and Sanchis, 2013). The Hemolysin II (HlyII) toxin is encoded by the *hlyII* gene. It induces macrophage apoptosis and allows *Bc* to bypass the host immune defenses (Cadot et al., 2010; Tran et al., 2011a; Tran et al., 2011a,b). Finally, emetic *Bc* strains induce the emetic food intoxication form through the production of a cereulide toxin (Yabutani et al., 2009). The toxin is synthesized by a non-ribosomal cereulide synthetase enzyme encoded by the plasmid-located *ces* gene (Marxen et al., 2015).

Epithelial cells are the primary physical barriers preventing microbes from entering the host. In addition, immune cells such as macrophages constitute a host defense against pathogenic bacteria. The heterogeneity of the diseases associated with *Bc* infections suggests that the ability of these bacteria to colonize their host, and to circumvent the host immune system may differ between strains, although the basis of these differences mostly remains unclear. In this work, we studied food-borne disease strains, strains isolated from human biological samples following local or systemic infections, and non-pathogenic strains, for their capacity to induce epithelial and macrophage cell toxicity, and we characterized the strains by the analysis of ten virulence genes revealing various genetic signatures.

2. Methods

2.1. Bacterial strains

This study included 39 *Bc* strains causing foodborne illness (Glasset et al., 2016), 41 strains isolated from human following systemic or local infection (Glasset et al., 2018) and 20 non-pathogenic strains, isolated from food that did not cause infection in humans or animals (Cadot

et al., 2010; Guinebretière et al., 2002; Kamar et al., 2013) (supplementary Table 1). The 39 strains isolated from foodborne illness and which caused gastrointestinal infections, were characterized in a previous study (Glasset et al., 2016). These strains were isolated between 2007 and 2014 in France. Each foodborne illness caused from 2 to 70 human cases, from 30 min to 24 h after ingestion of bacterial doses between 4.00E+02 and 1.00E+09 CFU/g of food. Identification and numeration of *Bc* strains from FBO were conducted by plating the strains on selective agar media (mannitol-phenol red-egg yolk medium (Biokar) according to the International Organization for Standardization (ISO) 7932 standard Method, allowing for *Bc* identification.

The 41 strains of the clinical collection were isolated from patient samples (biopsy, blood culture, etc) from nine French voluntary hospitals between 2008 and 2014 (Glasset et al., 2018). 41% of the patients were newborns and 21% died. *Bc* were identified by plating on specific agar media and confirmed by using 16S rDNA sequencing. The 20 non-pathogenic strains were isolated from soil or food that did not cause infections in humans (Guinebretière et al., 2002); They were also non-virulent in an insect infection model (Kamar et al., 2013).

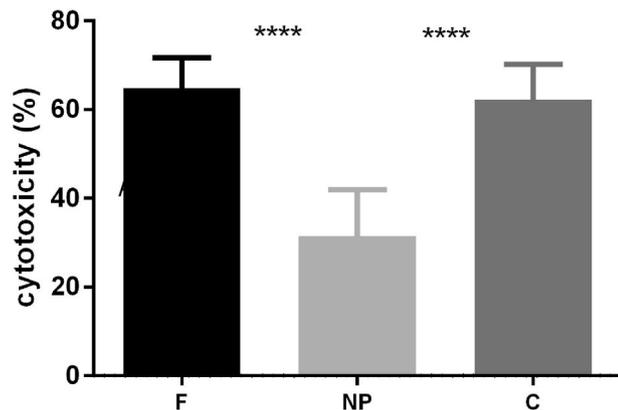
2.2. Cell lines and cytotoxicity assay

For cytotoxic studies, all strains were grown until the bacterial culture reached an optical density (OD) at 600 nm from 1.3 to 1.7, corresponding to the middle of the exponential growth phase. The cultures were centrifugated and supernatants were collected and filtered. The eukaryotic cells viability was assessed following incubation with bacterial culture supernatants. Tests were carried out on HeLa and Raw (Raw 264.7) cell lines (Sigma Aldrich).

Cell viability was measured by using the tetrazolium salt (MTS: (3-(4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2-(4-sulfo-phenyl) -2H-tetrazolium)) which colors the solution according to the mitochondrial activity. The eukaryotic cells were prepared in 96-well plates and incubated until confluence for HeLa and Raw cells. Cells were incubated at 37 °C + 5% CO₂ with specific media (HeLa: RPMI + 10% FCS + 2% penicillin/streptavidin, Raw: DMEM + 10% FCS + 2% penicillin/streptavidin). Cell medium was removed and the bacterial supernatants were incubated with eukaryotic cells at dilutions 1/10 (V/V in fresh cell medium) for 1 h.

Then, 20 µl of MTS were added and the OD was measured at 490 nm after 60 min of incubation at 37 °C + 5% CO₂. The percentage of cytotoxicity was calculated by normalizing the OD values between treated and untreated cells. Results represent the average of at least three experiments done in duplicates.

A.



B.

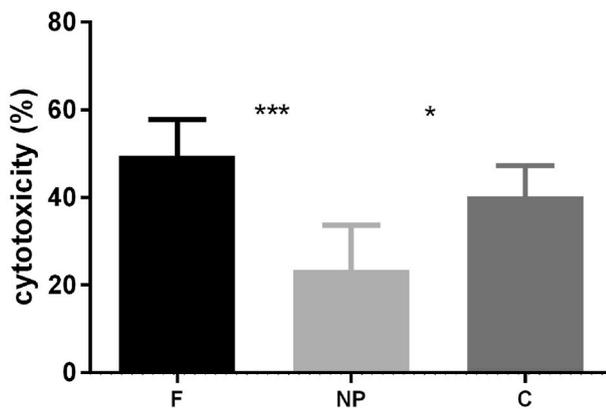


Fig. 1. Results of the toxicity of the bacterial culture supernatants belonging to the “foodborne illness”, “Clinical” and “non-pathogenic” collections. The percentage of cytotoxicity was calculated by normalizing the OD values (reflecting metabolically active cells) between treated and untreated cells. A.: Cytotoxic activity on the HeLa cell line. B.: Cytotoxic activity on the Raw cell line. Statistical analysis were performed and the significant difference is indicated as: **** $p < 0.0005$, *** $p < 0.001$ and * $p < 0.03$.

2.3. Molecular characterization

DNA was extracted after overnight incubation of the strains at 30 °C on Trypticase Soy Agar with 0.6 Yeast Extract (TSAYE, Sigma-Aldrich) using the DNeasy Blood and Tissue Kit according to the manufacturer’s recommendations (Qiagen). DNA was quantified by absorbance at 260 nm on a Nanodrop1000 spectrophotometer (Thermo scientific). The presence of ten virulence genes *cytK1*, *cytK2*, *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *hlyII* and *ces* was evaluated by PCR as described previously (Glasset et al., 2016). The combination of these genetic features allowed to attribute to each strain one genetic signatures (GS) (Table 1).

2.4. Statistical analysis

The cytotoxic activity results were analyzed by ANOVA and Tukey tests. ANOVA (analysis of variance) was used to assess whether all the Bc strain collections follow the same normal distribution. The Tukey test is a HSD test for “Honestly Significant Difference”, and was done after the ANOVA test. This paired comparison test compares each sample two by

two and identifies which ones differ from the normal distribution.

3. Results and discussion

3.1. Cell lines susceptibility

Each individual strain ($n = 100$) was tested for its cytotoxic potential to HeLa and Raw cell lines (sup Table 1). The average toxicity was then calculated for each collection of strains (Fig. 1). Bc strains involved in infections, foodborne illness or non-gastrointestinal infections, showed a greater cytotoxic activity than non-pathogenic strains on HeLa epithelial cells and mouse Raw macrophages. For HeLa cells, the difference is strongly significant ($p < 0.001$). On the Raw cell line, the difference is strongly significant between the foodborne illness strains and non-pathogenic strains (p value < 0.001) and significant between the clinical and non-pathogenic strains (p value = 0.023).

3.2. Genetic characterization

All strains from our collection were characterized by detecting by PCR the presence of these ten genes involved in virulence. According to the presence/absence of the genes, the strains were assigned a genetic signature (GS) (Table 1, Supp Table 1). Among all the possible combinations, all strains happened to spread in only twelve GS (Table 1). The strains were then grouped into three collections according to their induced pathologies (non-pathogenic, food-borne or clinical). We studied the distribution of GS in each collection (Table 1, Fig. 2). The distribution is not homogeneous. Six GS (GS1, 3, 6, 7, 9, 11) are present in the foodborne illness and clinical strain collections and are absent from the non-pathogenic collection. These signatures represent 52.4% of the foodborne illness strains and 55.4% of the clinical strains. Conversely, the GS8 signature is present only in the non-pathogenic collection (52% of the total NP strains). Four signatures are present in all collections (GS2, 4, 5 and 12).

GS1 represents 21% of the strains studied. This GS is the most frequent (34%) amongst the clinical strains. This genetic signature is characteristic of strains having only the genes encoding Nhe. However, 11% of the strains characterized by GS1 did not produce the toxin *in vitro* (not shown). Analysis must be carried out to understand what would explain the pathogenicity of these strains. A study has shown that the non-coding region upstream of the *nhe* operon promoter has more or less conserved regions. A binding site for the regulatory protein CodY has been identified and modifications in its sequence may explain the differences in regulation and expression of *nhe* (Bohm et al., 2016). Other factors may also be involved in the induction of clinical non gastro-intestinal symptoms.

GS3 and GS7 have the characteristics of the strains coding for the Cereulide and the Cytotoxin K1, respectively. These factors are known for their toxicity potential and their virulence on humans (Dierick et al., 2005; Lund et al., 2000; Mahler et al., 1997).

The *cytK1* carrying strains were found only in FBO strains and represented 9,5% of this collection. The GS3 strains, carrying *ces* and *nhe* genes were present in FBO (19%) but also clinical (14%) strains.

GS6, GS9, GS11 were present only in pathogenic strains and GS11 was even represented only among clinical strains. GS11 is characterized by the presence of *cytK2*, *ces* and *nhe* genes. To our knowledge, the role of the *ces* gene in clinical outcome has not been shown and further investigation are needed to correlate the pathogenic factors of these strains to their clinical outcome.

GS2 was found in 27% of the strains but was involved in one-third of the food borne outbreaks. This genetic signature is characteristic of strains possessing the genes encoding Nhe, Hbl and CytK2 and produce strongly Nhe and Hbl *in vitro*. A synergistic action of these toxins could explain its strong involvement in FBO (Beecher and Wong, 2000). However, this GS was also found in 19% of the non-pathogenic strains.

Interestingly, only one GS, GS8 corresponding to strains carrying

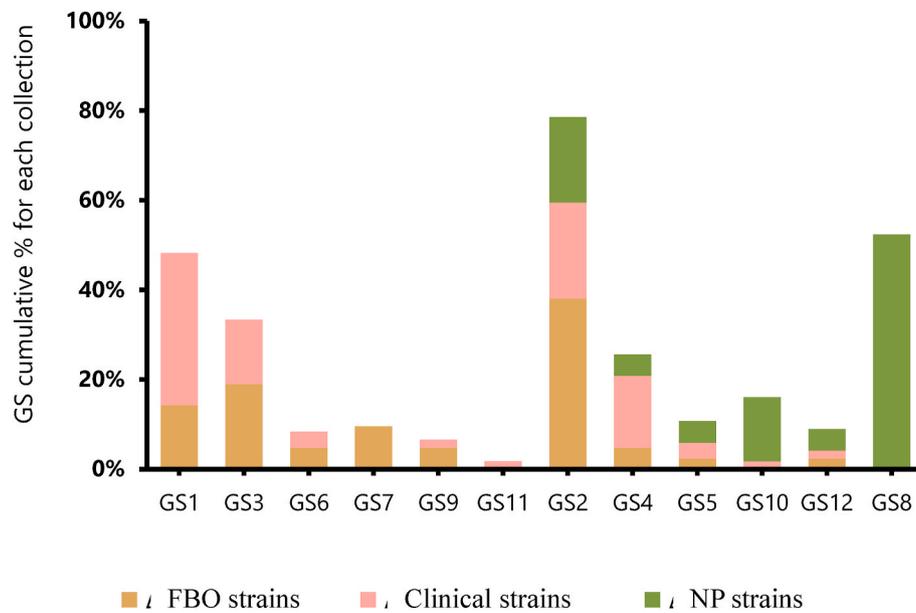


Fig. 2. Cumulative genetic signature (GS) distribution according to the “foodborne illness”, “Clinical” and “non-pathogenic” collections.

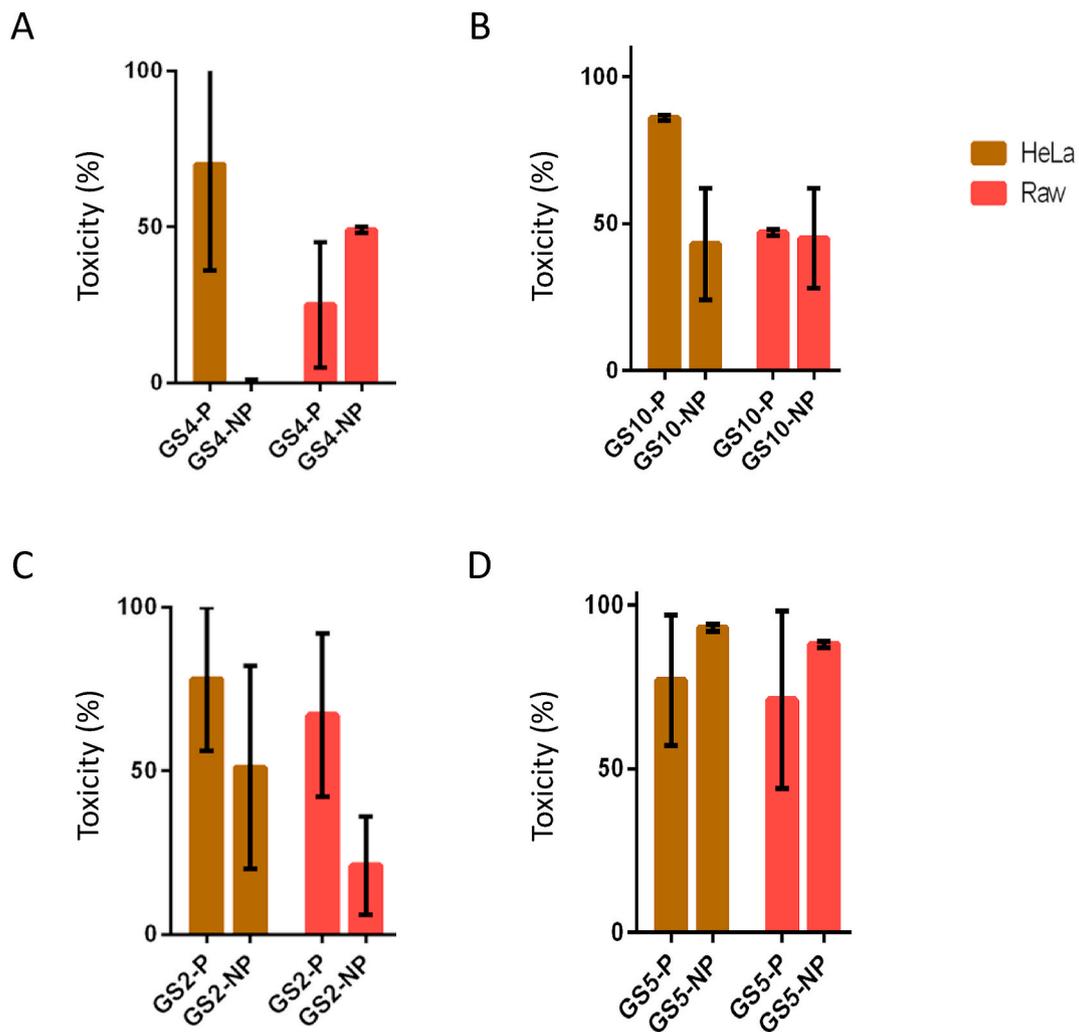


Fig. 3. Toxicity of culture supernatants of strains of *B. cereus* characterized by GS4 (A), GS10 (B), GS 2 (C) or GS 5 (D) and belonging to the pathogenic (-P) or non-pathogenic (-NP) collection.

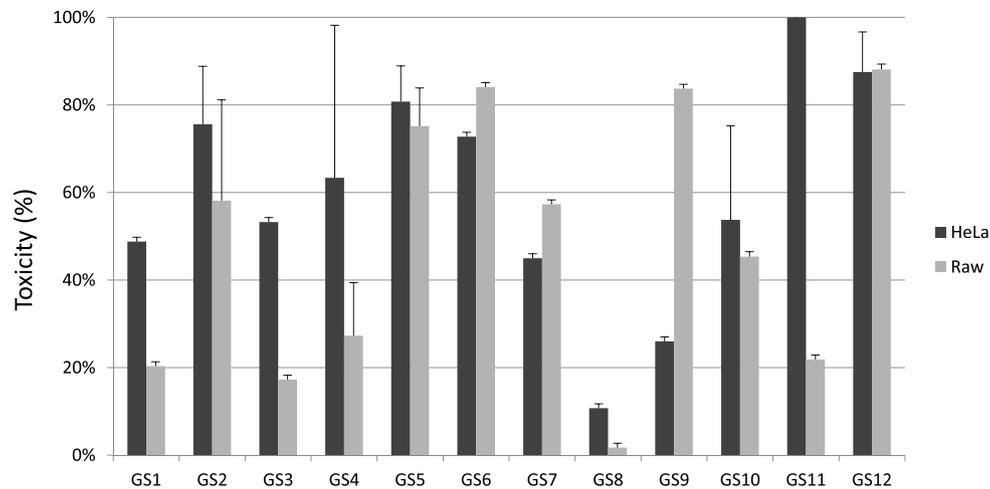


Fig. 4. Average toxicity of culture supernatants of strains of *B. cereus* belonging to the GS 1 to 12.

only *nheB* and *nheC* from the *nhe* operon and only *hblA* and *hblD* from the *hbl* operon were specific to non-pathogenic strains. This strongly suggests that the complete two operons need to be present in a strain to give a potential pathogenic potential and that absence of a complete protein impairs the strain virulence. Alternatively, this may indicate a specific role in toxicity of the NheA and HblC subunits. Characterization of the strains causing non-gastrointestinal infections by genetic signatures has shown that 10/12 (83.3%) of the signatures are the same as those found in strains causing FBO. Only the strains characterized by GS8, specific for non-pathogenic strains, and GS7 specific for FBO associated with CytK1 were not found in clinical strains.

In general, the assignment of strains according to their genetic signature has made it possible to show the genetic diversity of *Bc* strains, which can be involved in FBO and other clinical outcomes. However, this did not make it possible to answer all the questions that arise on the differences in virulence potential between the strains, or on their supposed synergistic action. It therefore seems essential to look for other specific markers of pathogenicity than those identified so far.

3.3. Genetic signature and cytotoxicity correlation

To further analyze the strains, the strains belonging to each GS were compared for their cytotoxic potential towards human cells (Fig. 3). The strains were divided in two groups: the non-pathogenic strains (NP) and the pathogenic strains (FBO + clinical). Remarkably, for the GS4 and 10, the pathogenic strains were significantly more cytotoxic to epithelial cells than the non-pathogenic strains. This was however not the case regarding the cytotoxicity to macrophage cells, indicating that the factors required for toxicity to epithelial and macrophage cells are different (Ramarao et al., 2005; Minnaard et al., 2004).

For the GS2, the same trend was observed for HeLa although not significantly and by contrast the cytotoxic potential of GS2 strains to Raw cell was significantly higher for pathogenic strains compared to non-pathogenic strains (pvalue <0.001), further highlighting a different tropism of the virulence factors for epithelial and macrophage cells. For other GS, such as GS5, no significant difference in cell toxicity was observed for the two collections of strains.

This is consistent with previous data showing the involvement of different factors depending on the cell targeted. For instance, the role of Nhe, Hbl and CytK2 has been shown in the toxicity to epithelial cells (Bohm et al., 2016; Bouillaut et al., 2005; Jeßberger et al., 2015; Ramarao and Lereclus, 2006), and HlyII is involved in the toxicity to phagocytic cells such as macrophage (Tran, Guillemet et al., 2011b).

We could consistently show that the strains characterized by GS5, GS6, GS9 and GS12 carrying the gene coding for HlyII are very toxic to Raw cells (82% average toxicity) compared to the strains characterized

by other GS (31% average toxicity) (Fig. 4).

Similarly, the strains with the GS in which the *cytK2* gene is present (GS2, GS4 and GS11) showed a higher toxic potential on epithelial cells compared to the other strains, i.e. 73 % versus 55%, as has already been described (Jessberger et al., 2015). The strains of GS8 having the genes coding for NheB, NheC, HblA and HblD, belong only to the collection of non-pathogenic *Bc* and are the least toxic on the two cell lines with an average toxicity of 13%.

Therefore, the significantly lower toxicity of the collection of non-pathogenic strains (Fig. 1) is largely due to the strains characterized by GS8. The non-pathogenic strains characterized by the other genetic signatures did not show differences in toxicity compared to the pathogenic strains sharing these same genetic signatures.

4. Conclusions

The emergence of *Bc* as a foodborne pathogen and as an opportunistic pathogen has intensified the need to distinguish strains of public health concern. Over the years, new methods have been developed with the leading principle to detect and distinguish *Bc* from others *Bacillus* group members by a time-saving and in-situ analysis (Ramarao et al., 2020). For example, Manzano et al., (2009), compared different molecular methods that use specific probes and primers as recognition elements to distinguish *Bc* from *B. thuringiensis* from different sources (food, clinical and bio-pesticide). Another method used to distinguish *Bc* from other members of the *B. cereus* group is the genotyping using high-resolution melting analysis (Antolinos et al., 2012). The use of multi-locus sequence data allowed identification of several clades within the *B. cereus* group. The classification of the strains according to their affiliation to a phylogenetic group (I to VII) also offers a first useful indicator of risk (Guinebretière et al., 2010).

The need to better assess the *B. cereus* pathogenic potential has also led to the development of several molecular tools for *Bc* toxin quantification including Nhe and Hbl (Ramarao et al., 2020). All these tests give indication on the genetic and toxin production of the strains but are not sufficient to accurately predict their pathogenic potential.

In this study, 12 genetic signatures (GS) were defined allowing to classify the strains of various origins from our collection. Cytotoxic activity tests showed significant differences between *Bc* strains that led to infections and that have higher toxicity than non-pathogenic strains. The combination of GS and cytotoxicity provides a next step in the strain characterization, with some GS being more associated with cytotoxic and pathogenic strains than others. A complementary method associating MLST, phylogeny and GS identification may be an innovative alternative to current characterization and differentiation methods.

Declarations of competing interest

None.

Acknowledgment

This work was supported by the European EJP Toxdetect grant from the European Union Horizon 2020 research and innovation program under Grant Agreement No 773830 and by the Comue Paris Saclay, France Idex Program n°CDE-2018-002323 . BG held an INRAE/ANSES fellowship.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.fm.2021.103759>.

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