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Novel insights into pathogenic *Bacillus cereus*

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List of Abbreviations

ANI	Average nucleotide identity analysis
ANN	Artificial Neural Network
AtxA	Anthrax activator gene
BACTH	Bacterial Adenylate Cyclase-based Two-Hybrid System
BAM	Bacteriological Analytical Manual
BCAA	Branched-chained amino acids
BcET	*Bacillus cereus* enterotoxin T
BLAST	Basic Local Alignment Search Tool
CDS	Coding sequence
Ces	Cereulide synthetase
CytK	Cytotoxin K
Eag	Encoding EA1 (extractable antigen 1)
EF	Edema Factor
EMSA	Electrophoretic Mobility Shift Assay
EntFM	Enterotoxin FM
FDA	U.S. Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GTP	Guanosine triphosphate
Hbl	Hemolysin BL
HEp-2	Human larynx carcinoma cells
HTH	Helix-turn-helix
HRM	High-resolution melting analysis
LF	Lethal Factor
LC-MS	Liquid Chromatography coupled to Mass Spectrometry
MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
Nhe	Non-hemolytic enterotoxin
NRPS	Non-ribosomal peptide synthetase
PA	Protective antigen
PC-PLC	Phosphatidylcholine phospholipase C

PCR	Polymerase chain reaction
PlcR	Phospholipase C regulator
PPTase	4'-phosphopantheteinyl transferase
rMLST	Ribosomal multi-locus sequence typing
qRT-PCR	Quantitative real-time PCR
RPC	Reversed Phase Chromatography
Sap	Surface array protein
SIDA LC-MS	Stable Isotope Dilution Assay Liquid Chromatography Mass Spectrometry
S-layer	Surface layer
SMase	Sphingomyelinase
TEII	Type II thioesterase
UPLC-MS/MS	Ultraperformance Liquid Chromatography Tandem Mass Spectrometry

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Authors Contributions

Manuscript 1

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Manuscript 3

Enterotoxin production of *Bacillus thuringiensis* isolates from biopesticides, foods, and outbreaks

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SJ: Conceived and designed the study, carried out the experiments, analyzed and interpreted the data, wrote the manuscript
EK: Carried out the experiments
NH: Carried out the experiments
PB: Contributed strains
SG: Contributed strains
MB: Performed the chemometric analysis of FTIR spectral data
MC: Contributed strains
RS: Carried out the experiments, contributed strains
ME-S: Conceived and designed the study, analyzed and interpreted the data, wrote the manuscript

Declaration

The work included in this PhD thesis with the title “Novel insights into pathogenic *Bacillus cereus*” was conducted according to the rules of good scientific practice as indicated by the guidelines of the University of Veterinary Medicine, Vienna.

Summary

The most prominent members of the *Bacillus cereus* group are *Bacillus cereus sensu stricto* known as the foodborne pathogen, the zoonotic pathogen *Bacillus anthracis* notorious for its disease anthrax, and *Bacillus thuringiensis* famous as being the most common biological pesticide worldwide. One common feature of these bacteria is their production of toxins including enterotoxins such as cereulide, insecticidal crystal toxins (cry toxins), hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), and anthrax toxin posing a high risk to public health. Another common feature among these *B. cereus* group bacteria is their high genetic similarity. In this context, it is important to differentiate, decipher and understand the microorganisms' distinct regulatory mechanisms of toxin and enterotoxin synthesis to accomplish correct attribution and develop effective prevention strategies.

The first and main part of the PhD thesis was to decipher the role of a novel plasmid-encoded PagR-like transcriptional regulator in the regulatory network of emetic *B. cereus.* The cereulide synthase gene cluster is located on the plasmid pCER270 in emetic *B. cereus* sharing its DNA-backbone with the plasmid pXO1 of *B. anthracis*, illustrating a central role in toxin synthesis. In emetic *B. cereus*, the transcriptional regulator PagR is a member of the ArsR/SmtB family, has a direct influence on the complex process of cereulide toxin synthesis similar to the homologous PagR of *B. anthracis* governing anthrax toxin synthesis.

The second part of the PhD thesis was the establishment of a novel method for the isolation, detection and purification of the emetic toxin cereulide of *B. cereus* by Reversed Phase Chromatography (RPC), a robust tool for microbiological and biochemical research laboratories.

The third part of the PhD thesis was the determination and comparison of enterotoxin production of various *B. cereus* group isolates, especially *B. thuringiensis*. This analysis showed that some biopesticidal *B. thuringiensis* strains may pose a risk to consumer health. Thus, *B. thuringiensis* is not only a pathogen of insects and a biopesticide but should also be considered a potential foodborne pathogen.

Since bacteria of the *B. cereus* group including *B. anthracis* challenge human and animal health, further research is needed in the context of the One Health concept to prevent outbreaks associated with foodborne as well as zoonotic pathogens.

1. Introduction

1.1 The *Bacillus cereus* group

At least 12 species belong to the *Bacillus cereus* group (*sensu lato*) of which *Bacillus cereus* (*sensu stricto*), *Bacillus thuringiensis, Bacillus anthracis*, *Bacillus cytotoxicus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus weihenstephanensis*, and *Bacillus toyonensis* are the most prominent ones (Guinebretière et al., 2008, 2013; Jiménez et al., 2013; Böhm et al., 2015; Liu et al., 2015). Based on the high homology of their 16S rRNA genes, the extraordinary relatedness of their core genomes, and their pathogenetic characteristics, several proposals recommended that the *B. cereus* group should be referred to one single species, comprising several genomospecies (Helgason et al., 2000; Jensen et al., 2003; Stenfors Arnesen et al., 2008; Bottone, 2010; Ehling-Schulz et al., 2011, 2019; Okinaka and Keim, 2016; Carroll et al., 2022). In particular*, B. thuringiensis, B. anthracis,* and *B. cereus* exhibit high level of sequence similarity, more than 99 % identity of their 16S rRNA, and can not be distinguished from each other, since for e.g. *B. thuringiensis* and *B. mycoides* only differ from each other and from *B. cereus* and *B. anthracis* by only four to nine nucleotides (Ash et al., 1991a, 1991b).

Some *Bacillus* species in this group gained more prominence as relevant pathogens, such as *B. anthracis,* the causative agent of Anthrax, also known as a potential biological warfare agent because of its high toxicity; *B. thuringiensis* as a pathogen of insects and as a biological pesticide, and *B. cereus* as an opportunistic pathogen causing food poisoning (Drobniewski, 1993; Helgason et al., 2000; Aronson and Shai, 2001; Turnbull, 2002; Ehling-Schulz et al., 2004; Messelhäußer and Ehling-Schulz, 2018).

B. anthracis and *B. cereus* are closely-related species with members of both species harboring plasmid pXO1 or pXO1-like plasmids, which are in other species of the *B. cereus sensu lato* group (Baillie and Read, 2001; Ehling-Schulz et al., 2006a, 2019; Rasko et al., 2007). Emetic *B. cereus* strains carry similar plasmids, such as the megaplasmid pCER270*,* which shares its backbone with plasmid pXO1 of *B. anthracis* (Baillie and Read, 2001; Ehling-Schulz et al., 2006b, 2015, 2019; Rasko et al., 2007). Recently, it was shown that there is no extant exchange of plasmids between *B. anthracis* and *B. cereus* but rather a vertical descent facilitating a better understanding of the pathogenomic evolution of *B. anthracis* and its plasmids (Pena-Gonzalez et al., 2018). Only the presence of insecticidal crystal toxins indicates the identification of

B. thuringiensis, whereas the absence of those toxins indicates *B. cereus,* allowing the differentiation on the basis of insecticidal crystal toxins located on plasmids (Schnepf et al., 1998; Ehling-Schulz et al., 2011; Johler et al., 2018). Both microorganisms, *B. thuringiensis,* and *B. cereus* were previously thought to be the same species due to the fact that a molecular and cultural differentiation by 16S rDNA sequencing and routine microbiological detection methods is not possible (Helgason et al., 2000; Ehling-Schulz and Messelhäusser, 2013; Johler et al., 2018). Furthermore, it was demonstrated that the application of various *B. thuringiensis* strains as biopesticides may not only be a risk to food safety but also a serious public health risk due to naturally occurring and commercial use of *B. thuringiensis* worldwide (EFSA, 2016; Chattopadhyay and Banerjee, 2018; Johler et al., 2018).

Bacillus cereus sensu stricto, an opportunistic human pathogen, is a gram-positive and catalase-positive, rod-shaped, motile, facultatively anaerobic, endospore-forming, toxin-producing microorganism, which has an ubiquitous occurrence in the environment (Drobniewski, 1993; Paananen et al., 2002; Bottone, 2010; Messelhäußer and Ehling-Schulz, 2018; Ehling-Schulz et al., 2019; Dietrich et al., 2021). The bacterium is recognized as a serious problem in food industry because of its highly adhesive endospores spread in the environment, persist for extended period of time and are resistant to heat, dehydration, starvation, physical stresses, aridity, pH-changes (Shinagawa, 1990; Ehling-Schulz et al., 2004, 2019; Rajkovic et al., 2008; Stenfors Arnesen et al., 2008; Bottone, 2010; Ehling-Schulz and Messelhäusser, 2013; Jessberger et al., 2020).

The major causative agent emetic *B. cereus*, which is commonly found in food and soil, causes diarrhea and emesis, which are part of the gastroenteritis diseases, and also severe extra-intestinal infections including septicemia, endocarditis, meningitis endophthalmitis, pneumonia (Drobniewski, 1993; Hoffmaster et al., 2006; Stevens et al., 2012; Messelhäußer and Ehling-Schulz, 2018; Ehling-Schulz et al., 2019; Dietrich et al., 2021; Kalbhenn et al., 2022). Based on data from animal experiments, it is thought that for the emetic syndrome, ingestion of 8 µg to 10 µg per kg of body weight of cereulide is necessary (Shinagawa et al., 1995; Jääskeläinen et al., 2003; Stenfors Arnesen et al., 2008). This intoxication then leads to vomiting, nausea, and abdominal cramps (Shinagawa et al., 1995; Jääskeläinen et al., 2003; Stenfors Arnesen et al., 2008). Thus, an intoxication with cereulide is accompanied by a short incubation time of 0.5 hours to 6 hours after food ingestion (Shinagawa, 1990; Ehling-Schulz et al., 2004).

In comparison, diarrheal symptoms are caused by 10^5 to 10^8 vegetative *B. cereus* spores or cells in contaminated food and are described with abdominal pain, cramps and diarrhea with a prolonged incubation time of about 8 hours to 16 hours – on average 12 hours – after consumption (Shinagawa et al., 1995; Jääskeläinen et al., 2003; Stenfors Arnesen et al., 2008). Notably, in different emetic *B. cereus* strains, the toxigenic potential varies from 100-fold up to 1,000-fold (Häggblom et al., 2002; Ehling-Schulz et al., 2005; Apetroaie et al., 2005; Carlin et al., 2006; Jovanovic et al., 2021).

1.2 Enterotoxins, the emetic toxin cereulide, and its biosynthesis

Three proteinaceous enterotoxins in *Bacillus cereus* named Nhe, Hbl, and cytotoxin K (CytK) are associated with diarrheal symptoms, whereas the emetic symptoms are associated with the heat-stable toxin cereulide (Beecher and Macmillan, 1991; Agata et al., 1995; Lund and Granum, 1996; Lund et al., 2000; Bottone, 2010; EFSA, 2016; Dietrich et al., 2021). In emetic *B. cereus,* most virulence factors are chromosomally encoded such as the diarrhea-causing enterotoxins, whereas the *ces* gene cluster is encoded on the pXO1-like megaplasmid, designated pCER270, which is closely related to the plasmid pXO1 of *B. anthracis* (Ehling-Schulz et al., 2006a; Rasko et al., 2007).

The emetic toxin cereulide is a 1.2 kDa small dodecadepsipeptide composed of alternating α-amino and α-hydroxy acid monomers $(D\text{-}O\text{-}Leu\text{-}D\text{-}Ala\text{-}L\text{-}O\text{-}Val\text{-}L\text{-}Val)_3$ with a 36-membered ring containing repeating ester and amide bonds (Agata et al., 1994, 1995). The chemical structure of the emetic toxin cereulide is very similar to that of valinomycin, an antibiotic which is produced by *Streptomyces* spp. (Figure 1), (Agata et al., 1994; Magarvey et al., 2006).

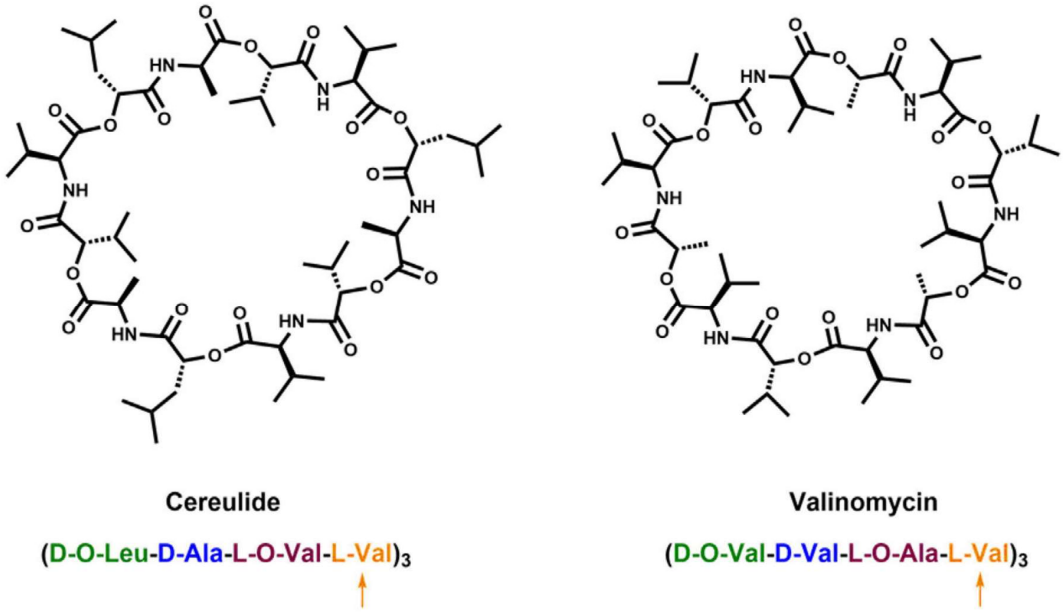

Figure 1: Chemical structure of cereulide and valinomycin. Amino acid differences are indicated in green, blue, and purple except for L-Val which is identical (indicated in orange). Picture taken from Jovanovic et al., 2021.

Valinomycin and cereulide act as a potassium ionophore leading to cellular damaging effects such as mitochondria damage, but cereulide has a much higher toxic potential compared to valinomycin (Agata et al., 1995; Shinagawa et al., 1995; Mikkola et al., 1999; Jääskeläinen et al., 2003; Ehling-Schulz et al., 2005; Stenfors Arnesen et al., 2008; Gopal et al., 2015). Based on its specific structure, cereulide is extremely resistant to pH values such as acid and basic conditions, and heat as well as proteolysis (Mikami et al., 1994; Shinagawa et al., 1995; Mikkola et al., 1999; Rajkovic et al., 2008; Stenfors Arnesen et al., 2008; Kalbhenn et al., 2021), and cannot be inactivated by standard hygienic procedures (Dietrich et al., 2021) in comparison to the heat-labile enterotoxins, which can be inactivated by high temperatures (Frenzel, 2012). It has been reported that cereulide is able to overcome the blood-brain barrier, as well as leading to swelling of mitochondria in human larynx carcinoma cells (HEp-2) and emesis in primates (Turnbull et al., 1979; Sakurai et al., 1994; Shinagawa et al., 1995; Bauer et al., 2018; Rouzeau-Szynalski et al., 2020). Cereulide and its effect on humans is a serious problem in the context of the One Health concept to prevent foodborne-related outbreaks.

The non-ribosomal peptide synthetase (NRPS), encoded by the polycistronic *ces* operon, synthesizes cereulide (Ehling-Schulz et al., 2005, 2006a; Magarvey et al., 2006; Dommel et al., 2010). The *ces* locus with a size of 24 kb consists of seven coding sequences (CDS) divided into the polycistronic transcribed *cesPTABCD* genes with a size of 23 kb and the individually transcribed *cesH* gene (Figure 2), (Ehling-Schulz et al., 2005, 2006a; Magarvey et al., 2006; Dommel et al., 2010). Two central promoters located upstream of *cesP* drive the transcription of the polycistronic cluster of the cereulide toxin genes, referred to the *ces* promoter P1 and P2, adding that the activity of promoter P1 was strongly nutrient dependent in real-time monitoring experiments measuring the *ces* promoter activity in various food samples (Dommel et al., 2010). The genes follow in order: *cesP* is responsible for the activation of NRPS and encodes a 4'-phosphopantheteinyl transferase (PPTase), (Dommel et al., 2010). The putative type II thioesterase (TEII) encoded by *cesT* has a proofreading function (Dommel et al., 2010). Located next to the genes *cesA* and *cesB* is the designated ABC transporter *cesCD* featuring a transport function essential for the NRPS multienzyme machinery (Dommel et al., 2010; Gacek-Matthews et al., 2020). In the experiments, the intracistronic promoters P_B and P_T were only weakly active (Dommel et al., 2010). Lastly, *cesH* encodes a putative hydrolase in its 5' region and is an additional regulator by acting direct or indirect as a transcriptional repressor in cereulide synthesis (Dommel et al., 2010; Lücking et al., 2015).

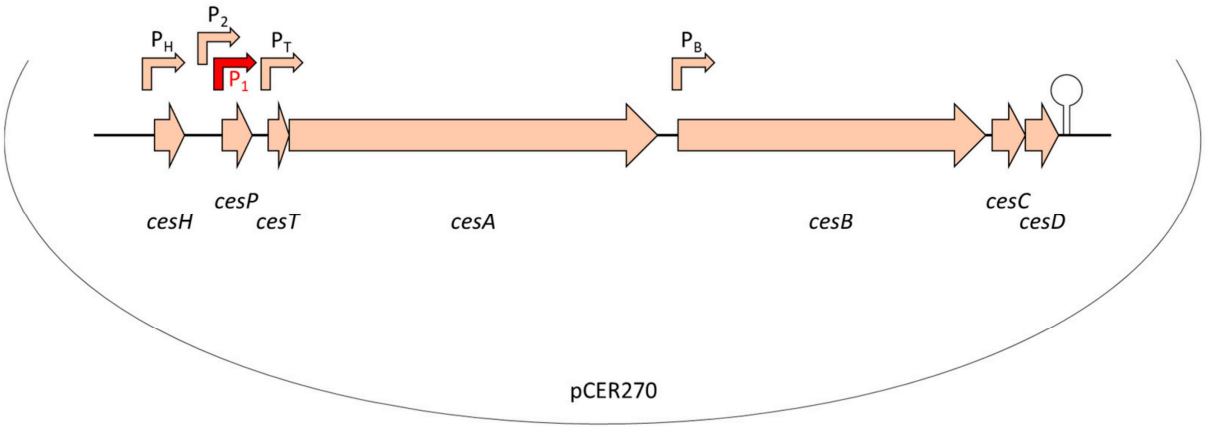

Figure 2: Schematic representation of the genetic organization of the 24 kb *ces* operon in emetic *B. cereus*. The *cesPTABCD* with a size of 23 kb is transcribed polycistronically, whereas *cesH* is transcribed individually with its own promoter. Arrows indicate promoters, respectively. The red arrow indicates the main *ces* promoter P1. The hairpin structure indicates the termination of the polycistronic *ces* operon. Illustration taken from Dietrich et al., 2021.

In emetic *B. cereus*, throughout the exponential phase and stationary phase, the *ces* transcription is regulated tightly and temporally in response to intrinsic and extrinsic factors (Dommel et al., 2011; Frenzel, 2012; Ehling-Schulz et al., 2015; Kranzler et al., 2016; Rouzeau-Szynalski et al., 2020; Jovanovic et al., 2021). Cereulide production depends on the availability of relevant factors such as temperature 12 °C to 40 °C, oxygen, carbohydrates, salt (NaCl) concentration, water activity, pH affecting the cereulide production and lead e. g. to a higher cereulide expression and accumulation in the late stationary growth phase (Agata et al., 2002; Ehling-Schulz et al., 2004; Dommel et al., 2010, 2011; Ehling-Schulz and Messelhäusser, 2013; Kranzler et al., 2016; Rouzeau-Szynalski et al., 2020).

1.3 The regulatory network of emetic *B. cereus*

The regulatory network of emetic toxin synthesis is a multilayered and complex process that depends on intrinsic and extrinsic factors resulting in a plasmid-chromosome crosstalk (Ehling-Schulz et al., 2015; Lücking et al., 2015). Since the *ces* gene cluster is located on the pXO1-like plasmid of emetic *B. cereus*, however, other transcriptional regulators are located on the chromosome and influencing the cereulide synthesis (Ehling-Schulz et al., 2015; Lücking et al., 2015).

The chromosome-encoded transcriptional regulators CodY and AbrB play a key role in earlier growth phases repressing the cereulide synthesis by binding to the *cesP* promoter regions (Lücking et al., 2009; Frenzel et al., 2012; Kalbhenn et al., 2022). Notably, AbrB is repressed by Spo0A, another chromosome-encoded regulator (Lücking et al., 2009; Frenzel et al., 2012), connecting the Spo0A-AbrB regulatory circuit and the developmental cell status to the cereulide synthesis (Lücking et al., 2009; Ehling-Schulz et al., 2015; Dietrich et al., 2021). The transcriptional regulator Spo0A is activated by the housekeeping σ^A factor having a role during the process of sporulation (Lücking et al., 2009; Frenzel et al., 2012; Ehling-Schulz et al., 2015; Jovanovic et al., 2021). A *spo0A* deletion mutant results in a specific phenotype, which is not able to produce cereulide (Lücking et al., 2009; Frenzel et al., 2012).

The global transcriptional regulator of cereulide synthesis CodY acts as an intracellular sensor and mediates transcriptional changes in response to the availability of nutrients after activation by guanosine triphosphate (GTP) and branched-chain amino acids (BCAAs), (Shivers and Sonenshein, 2004; Sonenshein, 2005, 2007; Barbieri et al., 2015; Belitsky et al., 2015; Ehling-Schulz et al., 2015; Dietrich et al., 2021). The nutrient-responsive master regulator CodY has been described to interlink the cereulide toxin synthesis and the enterotoxin synthesis with the primary metabolisms (Frenzel et al., 2012; Ehling-Schulz et al., 2015). The Spo0A phosphorelay leads to a repression of phospholipase C regulator (PlcR), whereas CodY activates the quorum sensing system PlcR/PapR operon and the PapR peptide activates PlcR (Declerck et al., 2007; Ehling-Schulz et al., 2015). The pleiotropic virulence factor PlcR is the regulator of for e. g. the quorum sensing mechanism and is well characterized in *B. cereus, B. anthracis* and *B. thuringiensis* (Gohar et al., 2002, 2008; Slamti and Lereclus, 2005; Pomerantsev et al., 2009; Sastalla et al., 2010; Slamti et al., 2014). PlcR does not control

cereulide at all but plays a major role in the regulatory network of enterotoxigenic *B. cereus* (Slamti and Lereclus, 2002, 2005; Gohar et al., 2008; Lücking et al., 2009; Slamti et al., 2014). The tight control of *ces* expression of emetic *B. cereus* must be influenced by other factors, since the transcriptional regulators CodY and AbrB repress cereulide synthesis only in the earlier growth phases (Kalbhenn et al., 2022). It was stated, that the timely control of cereulide toxin synthesis in the stationary phase is effected by the putative hydrolase CesH, which is an important member of the *ces* operon on plasmid pCER270 (Ehling-Schulz et al., 2006a; Dommel et al., 2010; Lücking et al., 2015; Kalbhenn et al., 2022). The putative repressor CesH, which is transcribed by its own promoter, degrades indirectly quorum-sensing molecules or metabolites, which are able to influence the cereulide synthesis in the later growth phases (Lücking et al., 2015).

In summary, besides the transcriptional regulators encoded on the chromosome, also regulators encoded on the megaplasmid pCER270 participate in the strict control of cereulide toxin synthesis (Ehling-Schulz et al., 2015; Lücking et al., 2015). However, mechanisms of the regulatory network of emetic *B. cereus* controlling the cereulide toxin synthesis and more importantly the interplay between the chromosome and the plasmid-encoded factors are hitherto unknown. Because of this, it is necessary to decipher the regulatory network of emetic *B. cereus* and reveal novel regulators that have a significant impact on cereulide toxin synthesis gaining new insights into the pathogenic *B. cereus*.

1.4 The plasmid-encoded transcriptional regulator PagR in *B. anthracis*

Bacillus anthracis is a gram-positive, catalase-positive, endospore-forming microorganism and the etiological agent of anthrax (Koehler, 2009). Over the last two decades, the virulence regulatory pathway in *B. anthracis* governing the anthrax toxins, have been studied in detail (Perego and Hoch, 2008; Fouet, 2010).

The anthrax toxins are major virulence factors of *B. anthracis*, located on the plasmid pXO1 (182 kb) and sharing its backbone with the pXO1-like plasmid designated pCER270 of emetic *B. cereus* (Welkos, 1991; Leppla, 2000; Koehler, 2002; Rasko et al., 2007). The virulence plasmid pXO1 harbors the genes *pagA* (encoding the Protective Antigen PA), *lef* (encoding the Lethal Factor LF), and *cya* (encoding the Edema Factor EF), as well as the major regulators

pagR and *atxA*, whereas the other genes are located on the second plasmid pXO2 encoding the biosynthetic operon *capBCADE* for the capsule (Makino et al., 1989; Okinaka et al., 1999; Koehler, 2002; Candela et al., 2005; Ehling-Schulz et al., 2019).

The master regulator in *B. anthracis* is the anthrax toxin activator gene AtxA, which regulates the toxin synthesis and capsule synthesis as well as the expression of the transcriptional regulator *pagR* (Uchida et al., 1993; Koehler et al., 1994; Dai et al., 1995; Hoffmaster and Koehler, 1999a; Drysdale et al., 2004; Perego and Hoch, 2008). The plasmid-encoded weak auto-repressor PagR mediates the expression of multiple virulence factors through a specific transduction cascade (Hoffmaster and Koehler, 1999a; Mignot et al., 2003; Kalbhenn et al., 2022). The transcriptional repressor PagR downregulates the *pagA* gene, whereas another transcriptional regulator designated PagR2 is located on the plasmid pXO2 and is involved in regulating the toxin gene expression of plasmid pXO1 in a specific manner (Hoffmaster and Koehler, 1999b; Zhao et al., 2010; Liang et al., 2016). The gene *pagR* can achieve a negative autogenous control of the *pag* operon (Hoffmaster and Koehler, 1999a; Mignot et al., 2003). The *pagAR* operon consists of two different transcripts: a larger mRNA transcript with a size of 4.2 kb encompassing *pagA* and *pagR* with an intergenic region of 0.9 kb and a smaller mRNA transcript with a size of 2.7 kb encompassing the *pagA* gene (Figure 3), (Hoffmaster and Koehler, 1999a; Mignot et al., 2003). Notably, the *pagR* gene is located 923 bp downstream of *pagA* in the same orientation (Hoffmaster and Koehler, 1999b). The transcription of the *pagAR* operon can be triggered by CO_2, as with the other virulence genes for e. g. *atxA* of *B. anthracis* (Mignot et al., 2003). The transcription of *pagAR* operon is induced by the major CO_2-inducible promoter P1 but has no detectable effect on the transcription from the minor promoter P2 (Figure 3), (Koehler et al., 1994; Hoffmaster and Koehler, 1999a; Mignot et al., 2003). For the transcription of the promotor P1, a trans-acting regulatory element located 13 kb upstream of *pag* is mandatory for the CO_2-enhanced transcription of *pag* (Koehler et al., 1994).

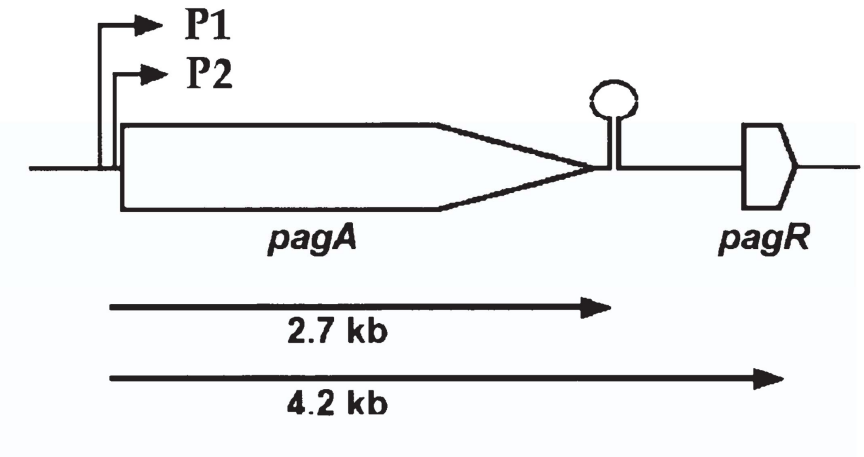

Figure 3: The genetic organization of the *pagAR* operon in *B. anthracis*. Arrows show two transcripts with a size of 2.7 kb and 4.2 kb. In between *pagA* and *pagR*, an intergenic region (0.9 kb) containing a stem-loop structure is indicated. Picture taken from Mignot et al., 2003.

The transcriptional regulator PagR binds to the promoter regions of the chromosome-encoded surface layer (S-layer) genes, *sap* (encoding the surface array protein Sap) and *eag* (encoding the extractable antigen 1 (EA1)), repress the transcription in case of *sap* and activate the transcription in case of *eag*; and fully accounts for an effect of AtxA on *sap* and *eag* (Fouet et al., 1999; Mignot et al., 2003; Fouet and Mock, 2006; Koehler, 2009). The transcription factors AbrB and SigH, located on the chromosome, are able to control the *atxA* gene, which regulates the *sap* and *eag* genes by the product of *pagA* co-transcribed by *pagR* (Hoffmaster and Koehler, 1999a; Perego and Hoch, 2008). It was hypothesized that *pagR* affects the expression of *pagA* in a feedback control loop as a result of *pagR* mimicking the expression of *pagA* (Hoffmaster and Koehler, 1999a). In addition, it was stated that *pagR* is able to repress the *atxA* expression by co-transcription with another *atxA*-activated gene indicating that *pagR* is the negative regulator of *atxA* (Hoffmaster and Koehler, 1999b).

The PagR protein is a DNA-binding molecule, whose amino acid sequence is similar to other members of the ArsR/SmtB family of metal-binding repressor proteins that act as transcriptional repressors (Xu et al., 1996; Busenlehner et al., 2003; Mignot et al., 2004; Zhao et al., 2010). Previously, the crystal structure of the transcriptional repressor of PagR of

B. anthracis was published, showing that PagR consists of five α-helices and two stranded β-sheets with a curved architecture and no metal binding sites (Zhao et al., 2010).

Since the plasmid-encoded transcriptional regulator PagR of *B. anthracis* has an impact on anthrax toxin synthesis and the entire regulatory network, there must be other PagR-like transcriptional regulators in emetic *B. cereus* that have an influence on cereulide toxin synthesis due to the close relationship between all members of the *B. cereus* group, which need to be deciphered.

2. Research objectives

Since knowledge and research on plasmid-encoded transcriptional regulators, which may influence cereulide toxin synthesis is lacking, the primary goal of this PhD thesis was to elucidate the role of plasmid-encoded transcriptional regulators located on the megaplasmid pCER270 in the regulatory network of cereulide toxin synthesis in emetic *B. cereus* strains. An in-depth understanding of this regulatory network is not only of scientific interest but could be crucial for the development of new effective strategies to prevent cereulide formation.

To achieve this goal, the work in this PhD thesis focused on the identification and characterization of a novel transcription factor named PagR (PagRBc) in emetic *B. cereus*, identified by *in silico* analysis showing homology to PagR (PagRBa), which is a key virulence regulator in *B. anthracis*. Initially, a deletion mutant designated F48Δ*pagR* was constructed and analyzed in detail using quantitative real-time PCR (qRT-PCR), Ultraperformance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS), Electrophoretic Mobility Shift Assay (EMSA) and a Bacterial Adenylate Cyclase-based Two-Hybrid System (BACTH).

Moreover, the Äkta™ pure system with RPC was adapted based on previous research by Tobias Bauer. It was used for cereulide detection and isolation from cultures of the isogenic F48Δ*pagR* mutant as well as from cultures of emetic or non-emetic representative strains of the *B. cereus* group, and cereulide-deficient isogenic mutants. This established approach provides a novel and widely accessible research tool for isolation, detection, quantification, and purification of cereulide complementing existing diagnostics.

Furthermore, an *in vitro* cytotoxicity assay was employed to investigate the enterotoxic activity of the F48Δ*pagR* mutant and other strains of the *B. cereus* group, including biopesticidal *B. thuringiensis*. The latter experiments were conducted within a cooperative project with the University of Zurich.

3. Publications

3.1 Manuscript 1

Impact of a Novel PagR-like Transcriptional Regulator on Cereulide Toxin Synthesis in Emetic *Bacillus cereus*

Eva Maria Kalbhenn, Markus Kranzler, Agnieszka Gacek-Matthews, Gregor Grass, Timo D. Stark, Elrike Frenzel and Monika Ehling-Schulz

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Article

Impact of a Novel PagR-like Transcriptional Regulator on Cereulide Toxin Synthesis in Emetic *Bacillus cereus*

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Abstract: The emetic type of foodborne disease caused by *Bacillus cereus* is produced by the small peptide toxin cereulide. The genetic locus encoding the Ces nonribosomal peptide synthetase (CesNRPS) multienzyme machinery is located on a 270 kb megaplasmid, designated pCER270, which shares its backbone with the *Bacillus anthracis* toxin plasmid pXO1. Although the *ces* genes are plasmid-borne, the chromosomally encoded pleiotropic transcriptional factors CodY and AbrB are key players in the control of *ces* transcription. Since these proteins only repress cereulide synthesis during earlier growth phases, other factors must be involved in the strict control of *ces* expression and its embedment in the bacterial life cycle. *In silico* genome analysis revealed that pCER270 carries a putative ArsR/SmtB family transcription factor showing high homology to PagR from *B. anthracis*. As PagR plays a crucial role in the regulation of the protective antigen gene *pagA*, which forms part of anthrax toxin, we used a gene-inactivation approach, combined with electrophoretic mobility shift assays and a bacterial two-hybrid system for dissecting the role of the PagR homologue PagRBc in the regulation of cereulide synthesis. Our results highlight that the plasmid-encoded transcriptional regulator PagRBc plays an important role in the complex and multilayered process of cereulide synthesis.

Keywords: *Bacillus cereus*; *pagR*; cereulide; transcription factor; homologue; *Bacillus anthracis*; toxins

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1. Introduction

Bacillus cereus is a major causative agent of two distinct forms of gastroenteritis diseases linked to food poisoning—emesis and diarrhea—as well as extra-intestinal infections, including endocarditis, endophthalmitis, and septicemia [1–5].

Most of the virulence factors of *B. cereus*, such as the diarrhea-causing enterotoxins, are located on the chromosome, while the *ces* genes – that are encoding the non-ribosomal peptide synthetase CesNRPS for the assembly of the emetic toxin cereulide—are located on a 270 kb plasmid pCER270. The megaplasmid pCER270 shares its origin of replication and its backbone with plasmid pXO1 from *Bacillus anthracis* [6,7]. The conserved state of pXO1 and pXO1-like plasmids is thought to reflect the relatively recent evolution of *B. anthracis* from a parental *B. cereus* subgroup [8]. The 182 kb pXO1 plasmid carries the anthrax toxin genes *cya*, *lef* and *pagA* as well as their major regulators *pagR* and *atxA* [2,9]. AtxA is known to act as the master virulence regulator in *B. anthracis*, exerting positive control on the anthrax toxin genes as well as the biosynthetic operon for the capsule synthesis,

encoded on a second virulence plasmid pXO2 [10–14]. The weak autorepressor *pagR*, which is co-transcribed with *pagA*, acts in a complex signal-transduction cascade that controls the expression of virulence factors [15,16]. PagR exerts its negative control on *pagA* by directly binding to the *pagAR* promoter and is also involved in the transcriptional regulation of the chromosomally encoded S-layer genes *sap* and *eag* [15].

While considerable progress has been achieved in dissecting the regulatory circuits of anthrax toxin production in *B. anthracis*, much less is known about the regulation of cereulide toxin biosynthesis in emetic *B. cereus*. The *ces* locus consists of seven coding sequences (CDSs), the individually transcribed *cesH* and the polycistronically transcribed *cesPTABCD* gene cluster [17–20]. *cesH* encodes a putative hydrolase, *cesP* encodes a 4′-phosphopanthetheinyl transferase (PPTase) responsible for the activation of CesNRPS (nonribosomal peptide synthesis), *cesT* is a type-II thioesterase (TEII) with a proofreading function, *cesAB* encodes the structural cereulide synthetase genes and *cesCD* encodes an ABC transporter, recently shown to be directly involved in the tethering of the CesNRPS to the membrane [21]. The polycistronic transcription of *cesPTABCD*, which is driven by the main *ces* promoter P1, is strictly regulated and tightly linked to the physiological status of the cell [17,22–24].

In previous studies, knockout mutagenesis has shown that the transcriptional regulator PlcR, which is known to play a central role in the pathology of enterotoxigenic *B. cereus* [25], was not involved in the regulation of the emetic toxin cereulide [26]. However, the synthesis of cereulide is controlled by the Spo0A phosphorelay [26] and CodY [22]. In the early growth stages, the chromosomally encoded AbrB binds to the *cesP* promoter region and represses the *ces* transcription until *abrB* transcription is ceased by Spo0A [26]. Furthermore, CodY has been described to act as a master regulator interlinking synthesis of the emetic toxin and enterotoxins with the general metabolism [22]. However, since CodY, as well as AbrB, are only repressing cereulide synthesis during the earlier growth phases, other factors have to be involved in the strict control of *ces* expression during the bacterial life cycle. Indeed, it has been reported that CesH, which is part of the *ces* locus, is taking part in the transcriptional control of cereulide synthesis [20,27]. Since *cesH* transcription is upregulated at a later growth phase than the *ces* operon, CesH may contribute indirectly to the shutdown of *ces* mRNA synthesis after entering the stationary phase by degrading metabolites or signaling molecules. The direct action of CesH as a transcription factor is unlikely because it encodes for a bona fide hydrolase [27].

In silico search for putative transcription factors encoded on the megaplasmid pCER270 in emetic *B. cereus* revealed two ArsR/SmtB family members, showing high sequence similarity with PagR from *B. anthracis*. The ArsR/SmtB family is widely distributed throughout non-pathogenic and pathogenic bacteria including *Staphylococcus aureus, Pseudomonas aeruginosa* or *Mycobacterium tuberculosis* [28]. Members of the ArsR/SmtB protein family act primarily as prokaryotic transcriptional repressors, which regulate the expression of genes associated with metal(loid) sequestration or efflux in Gram-positive and Gram-negative bacteria [29,30]. Originally, ArsR/SmtB family members were described as metalloregulators and named after their founding members, the regulatory protein ArsR of the plasmid-encoded arsenical resistance operon in *Escherichia coli* and SmtB, which represses the transcription of a class II metallothionein gene *smtA* in *Synechococcus* PCC7942 [31,32]. Despite the original described function of ArsR/SmtB family in metal sensing and metal homeostasis, other ArsR/SmtB family members show a broad variety of regulatory functions, such as in symbiosis, biofilm formation, stress response and virulence [28]. For instance, ArsR/SmtB transcriptional regulators involved in the control of virulence factors have been described in *Mycobacterium tuberculosis* (Rv2034), *Vibrio cholera* (HlyU) and *B. anthracis* (PagR), [33–35]. Despite their diverse regulatory functionality, the ArsR/SmtB family shows a common tertiary structure consisting of five α-helices (α1-α5) and a typical hairpin flanked by the two anti-parallel β-sheets (β1 and β2), which enable homodimer formation [30].

In this study, we aimed at deciphering the potential role of the two PagR homologous ArsR/SmtB family transcriptional factors located on the pCER270 plasmid for cereulide biosynthesis in emetic *B. cereus*. A gene-inactivation approach combined with electrophoretic mobility shift assays (EMSA) and a bacterial adenylate cyclase-based two-hybrid system (BACTH) allowed us to identify the first plasmid-encoded transcriptional regulator involved in the control of cereulide synthesis. This permitted us to gain further insights into the mechanisms orchestrating the interplay between chromosomally and plasmid-encoded factors controlling cereulide synthesis.

2. Results and Discussion

2.1. Identification of PagR-like ArsR/SmtB Family Regulators on the pCER270 Plasmid

We carried out an in silico analysis of the pCER270 sequence (GenBank accession number: DQ889676.1) to identify plasmid-encoded transcription factors potentially involved in the control of *ces* expression. We found two genes, BCAH187_RS28375 and BCAH187_RS28695, predicted to encode ArsR/SmtB family proteins, which we designated *pagRBc* and *pagR1Bc*, respectively. BCAH187_RS28375 and BCAH187_RS28695 are encoded on the sense strand, while des *ces* gene cluster *cesHPTABCD* is encoded on the anti-sense strand of pCER270 (see Supplementary Figure S1). The distance between the *ces* genes cluster and *pagRBc* is 102.5 kb, while *pagR1Bc* is located at a distance of 22 kb to the *ces* genes. Although *pagRBc* and *pagR1Bc* show sequence homology, there are no homologous genes found in their proximities (for details of the genetic *pagR* loci, see Supplementary Figure S2). However, a BlastP search revealed that PagRBc (YP_002335935.1) and PagR1Bc (ACJ82764.1) of emetic *B. cereus* show high similarity with *B. anthracis* PagR (PagRBa) and its homologues encoded on the *B. anthracis* virulence plasmids pXO1 (designated PagR1Ba) and pXO2 (designated PagR2Ba) (Figure 1A). The three-dimensional structure of PagR has been solved by multi-wavelength anomalous diffraction (MAD) and it was demonstrated that PagR bears the typical characteristics of an ArsR/SmtB family member, such as dimeric structure and a winged helix–turn–helix (HTH) DNA-binding domain, but lacks the classical metal-binding motifs [33]. A comparison of *B. anthracis* PagR and the PagR homologues of the emetic *B. cereus* and *B. anthracis* revealed that two specific protein motifs, "PQSTVSQHL" and "GLE", are conserved in all PagR homologues (Figure 1A). The first motif represents the DNA recognition sequence α-helix α4 (also known as αR), ensures contact with the major groove of DNA and mediates protein–DNA interactions. The α4 is highly conserved and characteristic for the ArsR/SmtB family members [28]. Further, the three residues S56, Q60 and L62 in α4 of PagR are thought to interact with the DNA major groove [33]. The second motif, "GLE", is located between the two β-sheets β1 and β2, forming a hairpin structure. Notably, the latter motif is also found in CadC, the transcriptional regulatory protein of the cadmium resistance system of *S. aureus* and *Listeria monocytogenes* [29]. *In silico* analysis of the protein sequences revealed that the residues, which are known to be crucial for DNA interactions of ArsR/SmtB repressors, are present in all PagR homologues (Figure 1A), indicating that these proteins are indeed ArsR/SmtB family members with yet to explore functionalities. With the exception of PagR2Ba, the residue Y81, which has been reported recently to be important for DNA binding of target gene promoters [36] and to be a potential tyrosine kinase phosphorylation site in PagR [16], is conserved in all PagR homologues. It is thus tempting to speculate that the PagR homologues share common molecular mechanisms.

Overall, PagR1Bc and PagR1Ba show the highest similarity among the PagR homologues, while PagR2Ba was found to be the most distantly related PagR homologue (Figure 1B). PagR1Bc and PagR1Ba differ only in four amino acid residues. Since these changes in the primary structure are located before or after the α-helices α1 to α5 and the β-sheets β 1 and β 2, it is tempting to speculate that both proteins are identical or very similar in their functional characteristics. The high similarity between PagR1Bc and PagR1Ba was also pinpointed by the results from a pairwise sequence alignment of PagR homologues performed by the Emboss Needle Algorithm to show an optimal sequence

alignment consisting of identity, similarity, score and gaps (Supplementary Figure S3). This analysis revealed an identity of 95.9% and a similarity of 99.0% of PagR1Bc to PagR1Ba, while PagRBc and PagR1Bc only share an identity of 54.5% and a similarity of 73.7%. Interestingly, PagRBc showed the highest identity (63.6%) and similarity (78.8%) to PagRBa, which indicates that PagRBc might be a functional homologue of PagRBa.

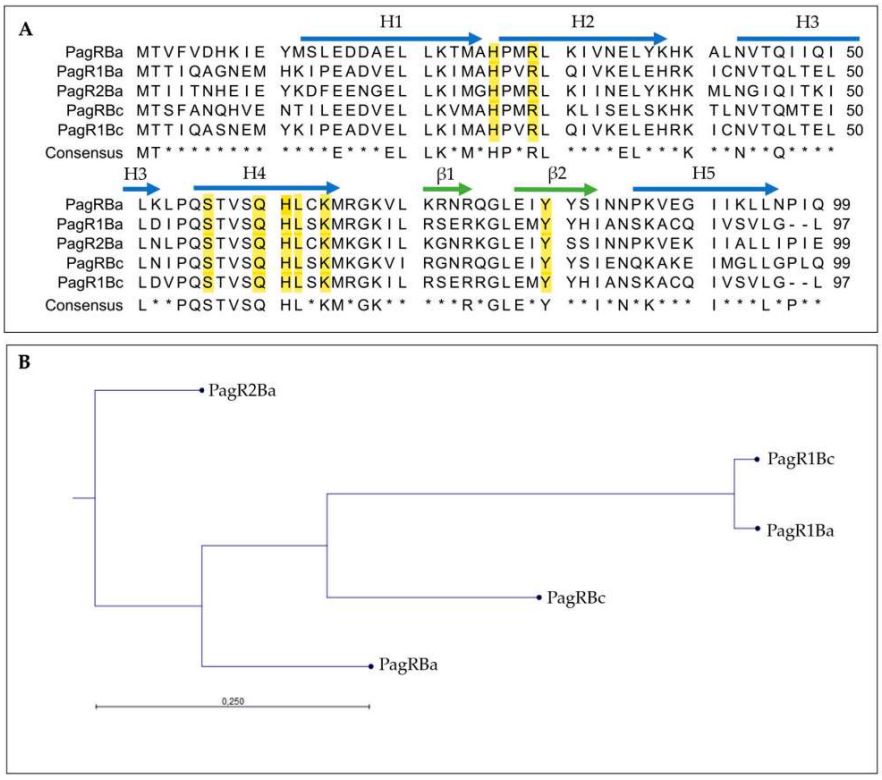

Figure 1. Amino acid sequence analysis of *B. anthracis* PagR (PagRBa) and its homologues on plasmids pXO1, pXO2 and pCER270. (**A**): Multiple sequence alignment of PagR homologues in emetic *B. cereus* and *B. anthracis*. The protein sequences of PagRBc (YP_002335935.1) from the emetic reference strain *B. cereus* F4810/72 and PagR1Bc (ACJ82764.1) were aligned to the two PagR protein sequences of *B. anthracis* Sterne encoded on pXO1 (PagR and PagR1) and the PagR homologue of *B. anthracis* Pasteur PagR2 encoded on pXO2 (for details of genome location, see Supplementary Figure S2). Sequences were retrieved from NCBI GenBank as follows: PagRBa (WP_000215715.1), PagR1Ba (AAD32441.1) and PagR2Ba (AJH31838.1). The consensus sequence is shown as identical amino acid residues in all PagRs in capital letters. Residues essential for DNA binding and physiological protein structure are highlighted in yellow [33]. Alpha helices (H1 to H5) are labelled with blue arrows and β-Sheets (β1 and β2) are labelled with green arrows. Asterisks * indicates amino acids not conserved in all sequences. (**B**): Protein similarity tree of the different PagR homologues based on the amino acid sequences of homologues from *B. cereus* (abbreviation: Bc) and *B. anthracis* (abbreviation: Ba) was calculated. The tree was constructed with the neighbor-joining method employing CLC Workbench Qiagen software. The Jukes–Cantor model was used for protein distance measurements.

We next employed the Phyre2 online tool [37] to construct predictive 3D models of PagR homologues, based on the crystal protein structure and a 3D model of PagR (PagRBa) of *B. anthracis* [33]. As depicted in Figure 2, all PagR homologues show similar 3D structures resembling the typical architecture of ArsR/SmtB family transcription repressors,

which leads to the unique structural folding of family members in the following order α1α2α3α4β1β2α5 [29,30]. However, while PagRBa, PagRBc and PagRBa2 are predicted to form all five α-helices (α1-α5) and the typical hairpin flanked by the two antiparallel β-sheets β1 and β2, the α1 helix seems to be only partially conserved in PagR1Ba and PagR1Bc.

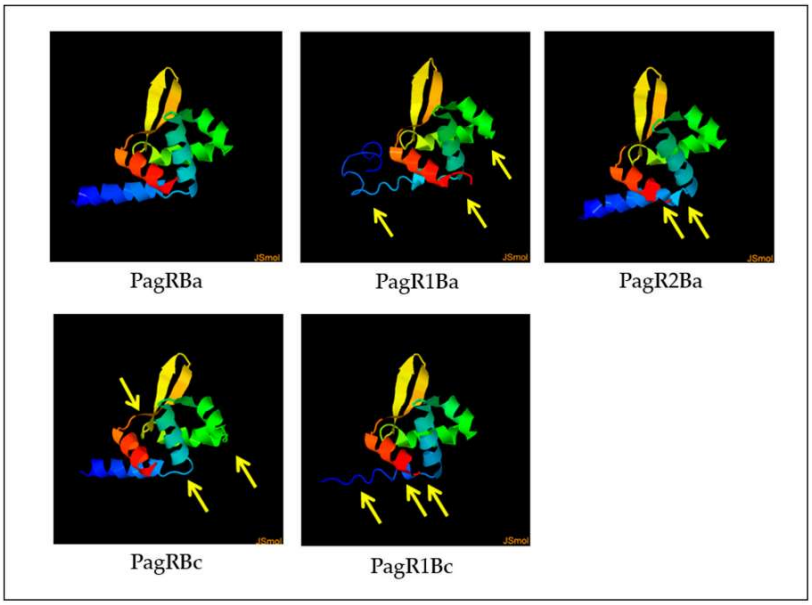

Figure 2. Predictive 3D protein models of PagR homologues. Structure models were constructed by the Phyre2 online tool, based on the crystal structure of PagR from *B. anthracis* [33]. Differences in protein folding are marked with yellow arrows of PagR homologues of emetic reference strain *B. cereus* F4810/72 and *B. anthracis* Sterne. Designations of PagR homologues as shown in Figure 1.

2.2. In Vitro Binding of PagR Homologues to the ces Promoter Region

Since PagR is a DNA binding protein that exerts negative control of *pagAR* transcription in *B. anthracis* by direct binding to the *pagAR* promoter [16], we investigated the binding affinity of His_6-tagged *B. cereus* PagR homologues (PagRBc, PagR1Bc) and the original His_6-tagged PagR from *B. anthracis* (PagRBa) to the main promoter region of the *ces* operon in emetic *B. cereus*.

For this, PagRBa, PagRBc, and PagR1Bc were heterologously expressed in *Escherichia coli* and the DNA-binding affinity of the purified proteins to the main *ces* promoter was tested by an electrophoretic mobility shift assay (EMSA). The promoter of the S-layer protein Eag, which is one of the main targets for PagR in *B. anthracis* [15], served as a positive control. Randomly amplified DNA fragments from emetic *B. cereus* F4810/72 served as a negative control. All three PagRs were able to bind the *cesP* probe containing the main *ces* promoter, while no specific interaction was observed within the unspecific DNA fragments (Figure 3). The highest binding affinity for the *ces* promoter was observed for PagRBc and PagRBa with an estimated equilibrium constant K_D of about 25 nM, whereas the binding of PagR1Bc was about six-fold weaker ($K_D \approx 150$ nM). Notably, PagRBa and PagRBc showed a comparable binding affinity for the *eag* promoter of *B. anthracis* and emetic *B. cereus* (K_D of 20 to 25 nM), whereas the binding affinity of PagR1Bc to the *eag* promoter was much weaker (Supplementary Figure S4). Furthermore, our in vitro binding studies revealed that PagRBa as well as PagRBc formed two distinct protein–DNA complexes of different sizes, with an increase of the slower migrating complex at higher concentration of PagRBc and PagRBa, respectively (Figure 3 and Supplementary Figure S4). Previously, it has been

reported that *S. aureus* CadC forms two protein–DNA complexes of different sizes. In line with our current findings, a shift to the higher molecular complex was observed with increasing amounts of CadC, possibly reflecting the binding of a second dimer to the DNA and the formation of a tetramer [38]. Although it has been reported that the binding of PagR dimers only covers 2.5 helical turns [33], it is known from DNase footprinting data that PagR binds to protect the DNA regions from the transcription at a length of nearly five helical turns [15]. Thus, it is tempting to speculate that PagR and its homologues may form tetrameric protein-DNA complexes to protect DNA regions from transcription *in vivo*. However, further studies, which are clearly beyond the scope of the current study, will be necessary to fully understand these highly dynamic and complex protein-DNA interactions.

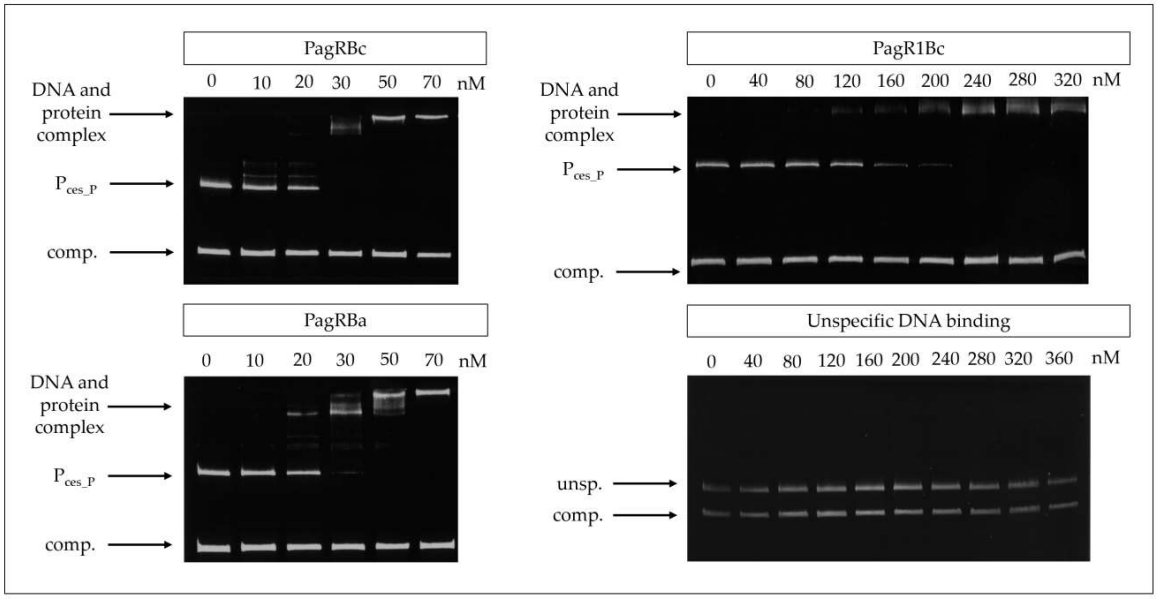

Figure 3. Gel mobility shift assay to determine the in vitro affinity of PagR homologues (PagRBc, PagR1Bc and PagRBa) to the *ces* gene promoter region of emetic *B. cereus*. The PagR homologues' potential to bind to the *ces* promoter (P_{ces_P}) was conducted in vitro using different amounts (0 nM up to 360 nM) of DNA comprising the promoter region of the *ces* operon or equimolar amounts of a competitive negative control DNA fragment (comp.), respectively. PagR concentrations are indicated in nM concerning the monomer on the top of each panel. Specificity of binding was further tested by using randomly amplified DNA from *B. cereus* (unsp.) with the competitive negative control DNA fragment (comp.) and increasing concentrations of purified PagR1Bc (lower right panel). A representative result from three independent experiments is shown.

Overall, our experiments demonstrate that PagRBc binds to the main promoter of the *ces* operon in vitro, suggesting that PagRBc may act as a transcriptional regulator of the cereulide synthetase gene cluster. Furthermore, the similar binding affinities of PagRBa and PagRBc for the *ces* and the *eag* promoter foster the hypothesis that these two proteins are not only structurally but also functionally homologues. Thus, we next invested their protein–protein interactions.

2.3. Interactions of PagR Homologues Identified by a Bacterial Two-Hybrid Assay

To test the ability of the *B. cereus* PagR homologues to form homodimers, which are the characteristic features of ArsR/SmtB family repressors [30], we used a bacterial adenylate cyclase-based two-hybrid system (BACTH) that allows testing protein interactions *in vivo* [39]. Both *B. cereus* PagR homologues were co-expressed with N-terminal and C-

terminal fusions to the adenylate cyclase (*cya*) fragments T18 and T25, respectively, and tested for the formation of blue colonies on X-Gal/IPTG agar plates, thereby indicating protein interactions. To determine the affinity of the protein interactions, the enzymatic activity of β-galactosidase was additionally quantified in bacterial extracts by using the Miller Assay [40]. As depicted in Figure 4, the self-interaction of PagRBc was about 1.75-fold stronger than the self-interaction of PagR1Bc. The comparably weaker self-interaction of PagRBc might be explained by the lack of a folded α-helix α1 (Figure 2). Helices α1 and α5 have been reported to build the core of the binding surface for PagR dimers [33], which is crucial for the correct positioning of the α4 (=αR) helix for protein–DNA interaction.

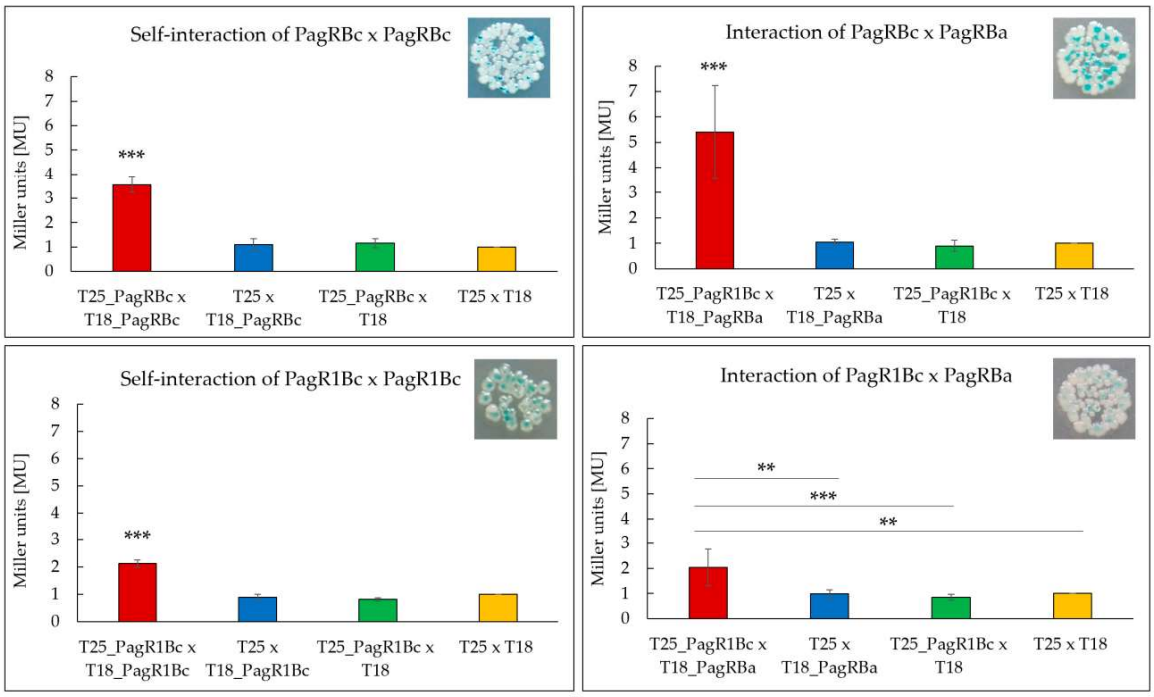

Figure 4. Screening of interacting PagR homologues of emetic *B. cereus* F4810/72 and *B. anthracis* Sterne strain by a Bacterial Two-Hybrid System Assay (BACTH assay). Self-interactions of the PagR proteins PagRBc and PagR1Bc are shown as well as the interactions of PagRBc × PagRBa and PagR1Bc × PagRBa. The efficacies of complementation between the indicated hybrid proteins were quantified by β-galactosidase assay (in Miller units [MU]). A color change to blue of the colonies indicates an interaction, respectively. Representative results from three independent experiments are shown, respectively. Statistically significant differences between the sample compared to the three controls are denoted as follows: ** $p < 0.01$, *** $p < 0.001$.

In addition, we expressed *pagRBa* fused to the N-terminal *cya* fragment T18 together with each of the *B. cereus pagR* homologues fused to the C-terminal *cya* fragment T25 to investigate whether the predicted structural homologies are also reflected in functional interactions *in vivo*. As expected, the PagRBa showed stronger interactions with PagRBc (about 2.5-fold) than with PagR1Bc, reflecting once more the strong structural homologies between PagRBa and PagRBc. Since PagRBa is known to repress the expression of the anthrax toxin component *pagA*, we next tested the effect of PagRBc on the synthesis of the cereulide toxin by generating a *pagRBc* gene-deletion mutant.

2.4. Phenotypic Characterization of a pagRBc Gene-Deletion Mutant

To assess the effect of PagRBc on cereulide biosynthesis, a single null mutant strain was constructed by allelic gene replacement of the *pagRBc* ORF in the emetic *B. cereus* reference strain F4810/72, resulting in the double-crossover *pagRBc*::*spc* strain F48Δ*pagR*. The growth of the *pagRBc* null mutant strain starts slightly later than the wild type but the increase in OD is similar (Figure 5A).

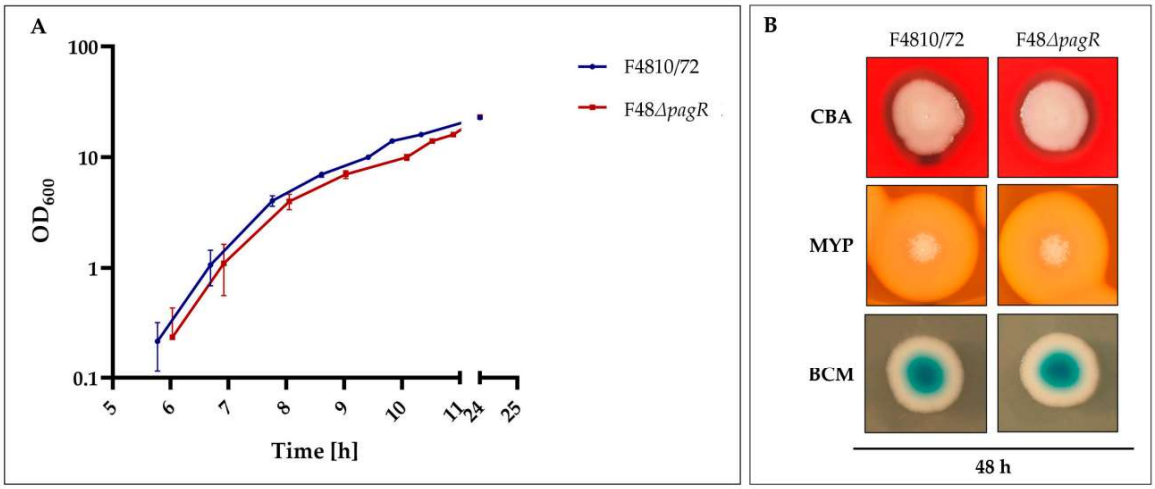

Figure 5. Growth and colony phenotypes of emetic *B. cereus* F4810/72 and its isogenic F48Δ*pagR* mutant. (**A**): Growth curves of wild-type and F48Δ*pagR* mutant. Strains were cultivated in LB broth at 30 °C. The growth was monitored by measuring the optical density at 600 nm (OD_{600}), shown are averages with standard deviations from three independent experiments. (**B**): Strains were grown on Columbia blood agar (CBA), Mannitol egg yolk polymyxin agar (MYP) and Brilliance *Bacillus cereus agar* (BCM), incubated at 30 °C for 48 h.

Furthermore, the F48Δ*pagR* and the wild-type strain F4810/72 did not exhibit differences in hemolysis nor in phosphatidylcholine- and phosphatidylinositol-specific phospholipases (PC-PLC and PI-PLC), as tested by growth at 30 °C on Columbia blood agar (CBA), Mannitol egg yolk polymyxin agar (MYP) and Brilliance *Bacillus cereus* agar (BCM), respectively (Figure 5B). These results suggest that PagR does not affect these chromosomally encoded virulence factors, which belong to the PlcR regulon [25].

2.5. PagRBc Acts as a Repressor of Cereulide Synthetase Gene Expression

To examine the impact of PagRBc on *ces* transcription, we performed qRT-PCR-based quantification of mRNA levels. To compensate for slight growth delay of the mutant compared to its parental, we isolated RNA from cultures sampled at the same OD_{600} and monitored *ces* transcription from OD_{600} of 0.2 to 16 (Figure 6A). While the expression of *ces* in the wild-type strain was restricted to the late exponential/early stationary phase, the absence of *pagRBc* resulted in an approx. four-fold enhanced synthesis of *ces* mRNA during the early growth phase (Figure 6B). Generally, the *ces* mRNA levels in the *pagRBc* null mutant were significantly higher during the different growth phases, although both strains reached the highest *ces* transcription levels around the same OD_{600}. In the wild-type *B. cereus* F4810/72, maximum *ces* transcription peaked at OD_{600} of 10 and declined thereafter, while the *ces* mRNA levels in the isogenic F48Δ*pagR* mutant stayed constantly high. These results indicate that the pCER270 encoded *pagRBc* acts as a transcriptional repressor of the cereulide toxin synthetase operon *ces*.

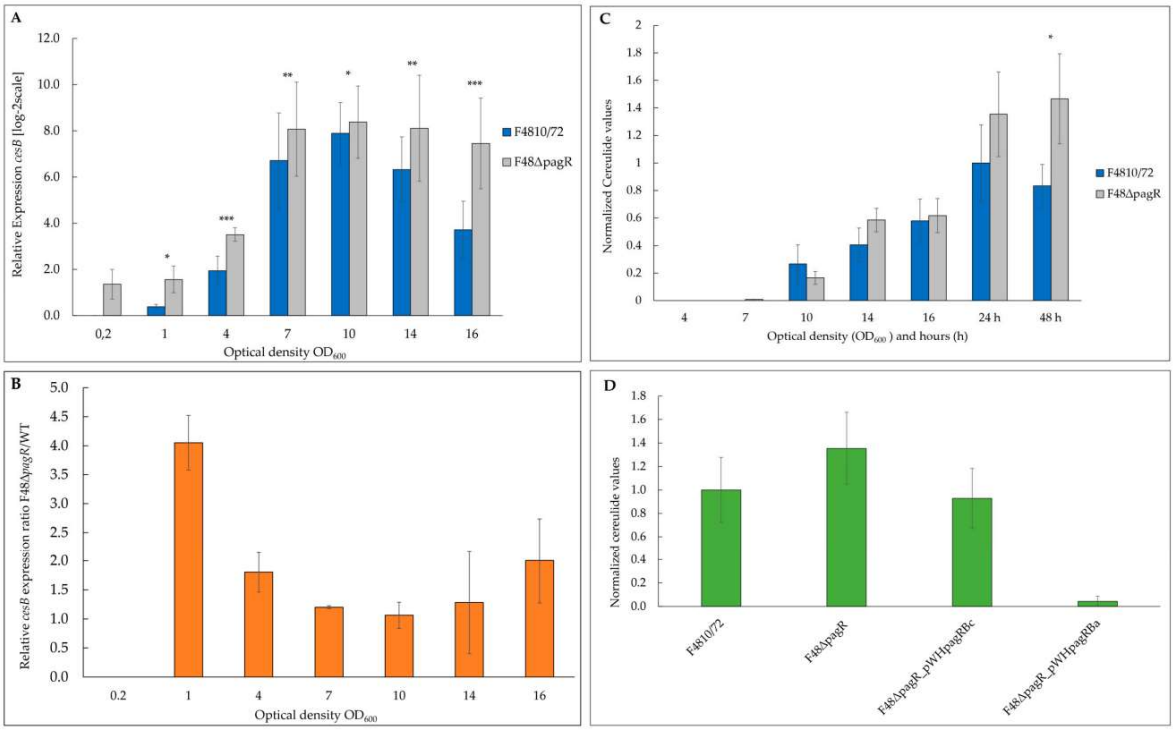

Figure 6. PagRBc represses cereulide toxin synthesis. The emetic reference strain *B. cereus* F4810/72 (wt) and its isogenic F48Δ*pagR* mutant were grown in LB broth at 30 °C. Total RNA was extracted from *B. cereus* F4810/72 and F48Δ*pagR*, harvested at indicated optical densities. Gene expression of *ces* and amounts of the cereulide toxin was determined at the OD_{600} as indicated. (**A**) The kinetics of *ces* transcription in F48Δ*pagR* and its wild-type parent were determined by qRT-PCR. Levels of *ces* expression were determined by qRT-PCR and normalized to 16S *rrn* levels of the same sample preparations. The expression level of *ces* gene obtained for the wild-type strain at an OD_{600} of 0.2 (external calibrator sample) was set to 1 (R = 1) by default, and all other expression levels were compared relative to this condition using the REST method [41]. Data were calculated from at least two independent qPCR measurements from three independent biological replicates. Statistically significant differences between the strains are denoted as follows: * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$. (**B**) Results of a comparative REST analysis of the relative *cesB* transcription levels of F48Δ*pagR* and the wild-type strain. The *rrn*-normalized *cesB* expression values in F48Δ*pagR* are depicted as log-2 ratios relative to the normalized *cesB* expression values of the wild-type strain at the same optical densities. Statistically significant differences in the *cesB* mRNA levels between the strains are indicated as denoted in (**A**). (**C**,**D**) The amount of cereulide in F48Δ*pagR*, the wild-type strain and the *pagRBc* complementation strains was determined by UPLC-MS/MS. (**C**) Samples from F48Δ*pagR* and the wild-type strain were analyzed at the OD_{600}s and hours indicated. (**D**) In addition, cereulide was determined in F48Δ*pagR* complemented *in trans* with *pagRBc* (F48Δ*pagR*_pWH*pagRBc*) and *pagRBa* (F48Δ*pagR*_pWH*pagRBa*), respectively; using a xylose-inducible promoter. Cereulide levels were calculated relative to the cereulide amount of the wild-type strain F4810/72 at 24 h. Samples were taken from three independent experiments shown as averages with standard deviations.

In line with the results from the *ces* expression studies, the cereulide toxin was detectable earlier (at an OD_{600} of 7) by ultra-performance liquid chromatography (UPLC) tandem mass spectrometry (MS/MS) in the mutant F48Δ*pagR* than in the *B. cereus* F4810/72 wild-type strain, and higher toxin levels were observed after 24 and 48 h (Figure 6C).

Since we found PagR (PagRBa) from *B. anthracis* not only to be structurally similar to *B. cereus* PagRBc but also to share functional characteristics with PagRBc, we examined if a heterologous PagRBa complementation strain could downregulate the increased cereulide production observed in the *pagRBc* null mutant. In parallel, we re-introduced *pagRBc* into F48Δ*pagR* to generate a homologous complementation strain, in order to prove whether the increased cereulide production indeed would result from the deletion of *pagRBc*. Thus, we amplified *pagR* from *B. anthracis* Sterne and *pagRBc* from *B. cereus* F4810/72, respectively, fused the genes to a xylose-inducible promoter in the *B. cereus* expression vector pWH1520 and introduced these plasmid constructs into the F48Δ*pagR* mutant. As expected, the induction of *pagRBc* with 0.1% xylose in the new strain—designated F48Δ*pagR*_pWH*pagRBc*—reduced the cereulide levels to those observed in the wild-type F4810/72 strain. Furthermore, the induction of *pagRBa* with 0.1% xylose in the *pagRBa* complemented mutant strain—designated F48Δ*pagR*_pWHpagRBa—led to a strong reduction of cereulide levels (Figure 6D). These results demonstrate that PagR plays a significant role in the regulation of cereulide biosynthesis. Furthermore, they show that besides *B. cereus* PagRBc, the structurally related PagR from *B. anthracis* substitutes the function of indigenous PagRBc in cereulide biosynthesis. The strong repressing effect of PagRBa on cereulide production observed in the heterologous complementation mutant indicates that PagR is able to form stable dimers, which mediate strong protein-DNA interactions.

Due to the high structural and functional homologies of PagRBa and PagRBc, we analyzed if the promoter regions of their target genes *eag* and *ces* comprise any conserved binding sites (see Supplementary Figure S5). However, as expected, no common binding site could be identified. Typically, ArsR/SmtB family members form dimeric or tetrameric proteins with a winged HTH-binding region and bind often imperfect inverted repeat motifs or imperfect palindromes [28,30]. Indeed, PagR (PagRBa) binds to DNA regions either symmetrically (to the *sap* promoter) or asymmetrically (to *pagA* and *eag* promoters, respectively), [15]. Thus, it is tempting to speculate that the *pagR* homologues in *B. anthracis* as well as in *B. cereus* follow a similar logic for binding to specific DNA regions. Another intriguing question that requires attention is the evolutionary origin of the PagR homologues encoded on the virulence plasmids of *B. anthracis* and emetic *B. cereus*, respectively. Although *B. cereus* pCER270 and *B. anthracis* pXO1 share a common backbone [7] and *pagR1Ba* and *pagR1Bc* are flanked by some homologous genes, the genetic organization of *pagRBa* and *pagRBc* is different (Supplementary Figure S2). There are no homologous genes found in the flanking genetic regions of *pagRBa* and *pagRBc* except for a Tn3 family transposase and its recombinase, which might be remnants from an ancient transition event. In contrast to *pagRBa*, which is known to be bicistronically transcribed with *pagA* [16], *pagRBc* is transcribed monocistronically, independently of the gene at its 5'-proximity, designated *hyp1* (see Supplementary Figure S6). Thus, further studies will be necessary to fully decipher the molecular functionality of PagRBc and its role in the virulence of emetic *B. cereus*.

3. Materials and Methods

3.1. Bacterial Strains and Culture Conditions

A list of all bacterial strains used in this study is provided in Table 1. If not indicated otherwise, *E. coli* strains were routinely grown in LB broth (LB) or on LB agar plates at 37 °C. The emetic reference strain *B. cereus* F4810/72 and its derivatives were grown at 30 °C in LB broth or on LB agar plates. For kinetic inoculation of the main cultures with a final inoculum of 10^3 CFU/mL of 100 mL fresh LB broth in baffled flasks, the emetic reference strain *B. cereus* F4810/72 and its derivatives were pre-cultivated for 16 h, as described previously [23,42]. Optical densities at 600 nm (OD_{600}) were recorded using a Biospectrometer Basic (Eppendorf, Hamburg, Germany). To remain within the linear absorption capacity of the instrument, samples exceeding an OD_{600} of 1 were measured as 1:10 dilutions in fresh growth medium and samples exceeding an OD_{600} of 10 were measured 1:100 diluted, respectively, and extrapolated. The following antibiotics were

added to the media when necessary: ampicillin (120 µg/mL), kanamycin (50 µg/mL) or 5-aminolevulonic acid (5ALA), [stock concentration: 50 mg/mL, final concentration 50 µg/mL) for *E. coli*; chloramphenicol (5 µg/mL) or tetracycline (10 µg/mL) for *B. cereus*.

Table 1. Bacterial strains used in this study.

Strain or Plasmid	Relevant Genotype or Characteristics	Reference or Source
E. coli		
E. coli TOP 10	General cloning host	Invitrogen
E. coli INV110	Methylase-deficient cloning host	Invitrogen
E. coli ST18	*E. coli S17lpir ΔhemA*	[43]
E. coli BL21(DE3)	Protein expression strain	Novagen
E. coli BL21 pET28b-*pagRBc*	Protein production strain for PagRBc used in EMSA	This study
E. coli BL21 pET28b-*pagR1Bc*	Protein production strain for PagR1Bc used in EMSA	This study
E. coli BL21 pET28b-*pagRBa*	Protein production strain for PagRBa used in EMSA	This study
E. coli BTH101 cya	Adenylate cyclase (*cya*) deficient reporter strain for bacterial two-hybrid screen (BACTH); F^-, *cya*-99, *ara*D139, *gal*E15, *gal*K16, *rps*L1 (Str^r), *hsd*R2, *mcr*A1, *mcr*B1	Euromedex
B. anthracis		
Sterne Strain	Vaccine strain devoid of pXO2 virulence plasmid	[44]
B. cereus		
F4810/72	Emetic reference strain, wild type; also termed AH187	[45,46]
F48Δ*pagR*	F4810/72 Δ*pagR*::cm; cm^r	This study
F48Δ*pagR_pWHpagRBc*	F48Δ*pagR* harboring pWH::*pagR* of emetic *B. cereus* [1] for PagRBc production, Tc^r	This study
F48Δ*pagR_pWHpagRBa*	F48Δ*pagR* harboring pWH::*pagR* of *B. anthracis* [2] for PagRBa production, Tc^r	This study

[1] Abbreviation of *B. cereus* is Bc. [2] Abbreviation of *B. anthracis* is Ba.

3.2. General Molecular Methods

DNA isolation, manipulation, transformation of *E. coli*, plasmid preparation, protein separation by SDS-PAGE and immunoblotting was carried out according to standard procedures [47,48]. For cloning purposes and generation of gel mobility assay samples as well as protein samples, genomic DNA of the emetic *B. cereus* F4810/72, and the *B. anthracis* Sterne strain served as a template. DNA amplification was carried out with Phusion High-Fidelity DNA Polymerase (Thermo Fisher, Waltham, MA, USA). The oligonucleotides (synthesized by Eurofins Ebersberg, Germany) used for plasmid construction and strain manipulation are listed in Supplementary Table S1. An overview of plasmids used is provided in Supplementary Table S2. All constructs were verified by restriction enzyme digest and Sanger DNA sequencing (LGC Genomics, GmbH, Berlin, Germany). *E. coli* strains were transformed by heat shock and *B. cereus* strains by electroporation as described previously [19]. Conjugation was carried out according to Thoma and Schober [43] as described below (see Section 3.7).

3.3. Construction of E. coli Protein Expression Strains for Electrophoretic Mobility-Shift Assay (EMSA)

For recombinant protein expression, DNA fragments encoding *pagRBc* and *pagR1Bc* were amplified from genomic DNA of emetic *B. cereus* F4810/72 by using the following primer pairs: PagRNco_F/PagRXho_R and PagR1Nde_F/PagR1Xho_R (Supplementary Table S1). DNA fragments encoding *pagR* of *B. anthracis* Sterne strain were amplified from genomic DNA using the primer pairs: PagRBaNco_F/PagRBaXho_R (Supplementary Table S1). Probes were designed to achieve N-terminal or C-terminal His_6-tag. Start and Stop codons were integrated and/or replaced in/with the respective restriction site. The amplified, digested and purified fragments were cloned into the expression vector pET28b(+). Constructs were verified by PCR and Sanger DNA-sequencing using the primers pET28b_for and pET28b_rev. For protein overexpression, the plasmids were introduced into *E. coli* BL21 (DE3) by transformation.

3.4. Production and Purification of Heterologous Proteins PagRBc-His$_6$, PagR1-His$_6$ of Emetic B. cereus and PagR-His$_6$ of B. anthracis

Proteins were produced with *E. coli* BL21 (DE3) as soluble, N-terminal or C-terminal His$_6$-tag fusions as previously described [22]. Protein production was induced by adding 1 mM IPTG to exponentially growing cells with an OD_{600} of 0.6. After 3 h incubation, cells were harvested at 6000× *g*, at 4 °C, 10 min and washed twice with washing buffer A [50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 0.5 mM Pefabloc (Roth, Karlsruhe, Germany)]. For protein purification, cells were resuspended in 3 mL Ni-NTA Native Lysis Buffer [50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8] containing 5 mM Pefabloc (Roth, Karlsruhe, Germany) and 3 µL of Benzonase [20,000 units], (Thermo Fisher, USA). Cells were disrupted twice with a French press (1 kbar) and cellular debris was removed by two times 20 min centrifugation step at 15,700 rpm at 4 °C. The supernatant was loaded onto Ni-NTA affinity columns (Qiagen, Chatsworth, CA, USA) pre-equilibrated with lysis buffer. The column was washed with 10 column volumes (CV) of washing buffer [50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0] and the protein was eluted in elution buffer [50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0]. The eluted fractions were dialyzed with the 10-fold BS buffer [50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM Na_2EDTA, pH 8.0, 10% glycerol] using ultrafiltration columns with a 10 kDa cut-off (Vivaspin 500 concentrators (Sartorius AG, Goettingen, Germany)). Protein purity was analyzed by SDS-PAGE and total protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermo Fisher, MA, USA), using bovine serum albumin as a standard.

3.5. Gel Mobility Shift Assay

Affinities of His$_6$-tagged transcription factors PagR, PagR1 of *B. cereus* F4810/72 and PagR of *B. anthracis* Sterne to the promoter region of the *ces* gene were analyzed with nonradioactive native PAGE in gel mobility shift assays [49]. To analyze the binding to the *ces* promoter, a 523 bp fragment covering the main promoter region of *cesPII* [17], the *ces* promoter was amplified with the primer pairs: cesPII_for and cesPII_rev (Supplementary Table S1). The binding reactions contained increasing amounts (0 ng up to 360 ng) of PagR-His$_6$, PagR1-His$_6$ of *B. cereus* F4810/72 and PagR-His$_6$ of *B. anthracis* Sterne and the *cesPII* promoter fragment in binding buffer [50 mM Tris-HCl (pH 8.0), 750 mM KCl, 2.5 mM Na_2EDTA (pH 8.0), 0.5% Triton X-100, 62.5% glycerin (*v*/*v*), 1 mM dithiothreitol (DTT)]. To monitor nonspecific binding, equimolar amounts of a randomly amplified 301 bp DNA fragment (EMSAunspec7), originating from the *cesH* coding sequence of *B. cereus* F4810/72, served as competing DNA as described previously [26]. To further test the specificity of DNA-binding, PagR1-His$_6$ was incubated with EMSAunspec7 and a second randomly amplified 201 bp DNA fragment (EMSAunspec8), origination from the *cesP* coding sequence of *B. cereus* F4810/72. The binding reaction was carried out as described above. Samples were incubated at RT for 30 min before being loaded onto a 10% native polyacrylamide gel which was run in pre-chilled TBE buffer at 120 V for 3 h at 4 °C. Gels were stained in ethidium bromide solution and visualized by UV irradiation.

3.6. Bacterial Two-Hybrid System (BACTH) and β-Galactosidase Assay

The genes *pagR1Bc* (NCBI locus tag: BCAH187_RS28695) and *pagRBc* (NCBI locus tag: BCAH187_RS28375) of the emetic *B. cereus* F4810/72, located on the pCER270 megaplasmid, and the *pagRBa* gene (NCBI locus tag AW20_RS00175) of *B. anthracis* (located on the pXO1-plasmid) were amplified (Supplementary Table S1) and cloned into the pKT25, pKNT25, pUT18 and pUTC18C BACTH expression vectors (Cat no: EUK001, Euromedex, Souffelweyersheim, France), (Table 1). The cloning procedure of the BACTH and the β-galactosidase assay were performed as recently described [21]. The respective plasmids were introduced into *E. coli* BTH101 *cya*-host strain by heat-shock transformation. The procedure is based on the functional complementation of two subunits of the adenylate cyclase (Cya) T18 and T25 fused with the putatively interacting partners as previously

described [39,50]. Each protein was tagged on the N- and C-terminus by both subunits of Cya (T18 and T25). For each putative interaction, all possible combinations were tested in order to assess homo- as well as heterodimers by cotransforming plasmid constructs into *E. coli* BTH101 cells and plating them on LB X-gal/IPTG agar and LB IPTG for ß-galactosidase assay. The cells were incubated for 24 h at 30 °C, followed by incubation for 20 h at RT and for 20 h at 18 °C. For the β-galactosidase assay, cells were harvested from the LB-IPTG plates and resuspended in 1 mL Z-buffer (10 mM KCl, 10 mM $MgSO_4$, 0.27% β-mercaptoethanol, Na-phosphate butter with pH of 7). The cell densities at OD_{600} were adjusted to 0.4 to 0.7, cells were permeabilized using β-mercaptoethanol, SDS and chloroform. The enzymatic reaction was carried out at 28 °C and started by adding 4 mg/mL ortho-Nitrophenyl-β-galactoside-sodium-phosphate buffer. After the samples turned yellow, the reactions were stopped with 1 M Na_2CO_3. The ß-galactosidase assay was carried out according to [50] and values were expressed in Miller Units [MU].

3.7. Construction of the B. cereus pagRBc Null Mutant Strain F48ΔpagR

To construct a *pagRBc* deletion mutant of the emetic *B. cereus* reference strain F4810/72, DNA regions of approximately 1500 bp flanking the *pagRBc* gene (NCBI locus tag: BCAH187_RS28375) were amplified with the primer pairs: pagRFl1_F/pagRFl1_R and pagRFl2_F/pag2Fl2_R for the flanking regions of *pagRBc* gene from emetic *B. cereus* F4810/72 DNA (Supplementary Table S1). The fragments were digested with KpnI/SacI and XhoI/XbaI, respectively. The amplification of a chloramphenicol-resistance cassette (1200 bp) from plasmid pAD123 [51] was carried out with the primer pairs CmEcoRI_F/CmEcoRI_R. The remaining fragment and the pCR 2.1 TOPO plasmid were digested with EcoRI and ligated by heat-shock transformation in *E. coli* TOP10 cells to result in pCR 2.1 TOPO/Cm construct (control primers for the insert were M13-F/M13-R). Both flanking region fragments were cloned into the chloramphenicol cassette containing plasmid pCR 2.1 TOPO/Cm. The construct was cut (KpnI/XbaI) and cloned into the similarly digested conjugative suicide pAT113 plasmid [52]. This plasmid was introduced into a diparental mating system with *E. coli* ST18, which was used to replace *pagRBc* with the chloramphenicol cassette in emetic *B. cereus* F4810/72 as described previously [43]. In brief, the diparental mating system is performed with *E. coli* ST18 strain, a *hemA* deletion mutant defective in tetrapyrrole biosynthesis, where the *hemA* mutation can easily be complemented by the addition of 5-aminolevulinic acid (5-ALA). The counterselection of the mating system is carried out by standard media and optimal growth conditions for the recipient strains. The conjugation was performed with the emetic *B. cereus* wild-type strain F4810/72 to yield the null mutant, designated F48Δ*pagR*. Gene deletion and integration of the resistance cassette was confirmed by PCR and sequencing using the primer pairs: pagRK1_F/pagRK1_R and pagRK2_F/pagRK2_R.

3.8. Expression of pagR Homologues in the pagRBc Null Mutant F48ΔpagR

To complement the *pagRBc* null mutant with *pagR* homologues from *B. cereus* as well as with *pagR* from *B. anthracis*, the respective genes were amplified from DNA of *B. cereus* F4801/72 and *B. anthracis* Sterne using the primers listed in Table S1. and introduced into pWH1520 plasmid to obtain pWH::*pagRBc* and pWH::*pagRBa*, in which the expression of *pagR* homologues is under control of a xylose-inducible promoter [53]. The plasmids were passaged through the non-methylating *E. coli* strain INV 110 and introduced into F48Δ*pagR* by electroporation. The successful uptake of the plasmids was verified by by PCR and subsequent sequencing of the PCR fragments with the primer pairs pWH1520_F/pWH1520_R (Supplementary Table S1). To induce the expression of the *pagR* homologues in F48Δ*pagR*, the cultures were kinetically inoculated 10^3 CFU/mL as described above and induced with D-xylose to a final concentration of 0.1% (*v*/*v*), respectively, harvested at 24 h for determination of cereulide toxin levels.

3.9. Transcriptional Analysis of cesB Expression by qRT-PCR

Transcription of the *cesB* gene in the *B. cereus pagRBc null mutant* F48Δ*pagR* and its parental strain was analyzed by qRT-PCR as described previously [23,42]. For RNA isolation, 2 mL to 5 mL bacterial culture was harvested (10,000× *g*, 4 °C, and 2 min) at different optical densities. The supernatant was discarded and the cell pellets were directly frozen using liquid nitrogen. Pellets were stored at −80 °C until RNA extraction. RNA was isolated from frozen cell pellets via TRIzol Reagent (Invitrogen, Thermo Fisher, USA) and homogenized with FastPrep®-24 Ribolyser of MP (2 times of 45 s, in between samples were chilled on ice for 30 s, speed 6.5) using 0.1 mm ZnSn beads in 2 mL screw-top tubes. Phase separation was carried out with chloroform and nucleic acid was precipitated with 75% ethanol. RNA concentration was measured by Nanodrop™ 2000 spectrophotometer (Thermo Fisher, USA). Samples were diluted in a ratio 1:10 or 1:100, respectively, to get 1 µg final volume for cDNA synthesis with iScript™ gDNA Clear Synthesis Kit (Bio-Rad Laboratories, Vienna, Austria). The cDNA was diluted in a ratio 1:25 for qRT-PCR performance. For qRT-PCR analysis, we used SSO Advanced Universal SYBR Green Supermix (Bio-Rad Laboratories, USA) according to manufacturer instructions. For each reaction, 10 µL of SYBR Green, 2.5 µL of primer_F (3 µM) and 2.5 µL primer_R (3 µM) was used. Primers for qRT-PCR are listed in Table S1. To every qRT-PCR reaction mix, 5 µL of 1:25 diluted cDNA was added (final volume of 20 µL). Reactions were run on Bio-Rad Cycler (Bio-Rad Laboratories, USA, CFX96 Real-Time System C1000 Touch). To monitor gene expression, the REST method (Relative Expression Software Tool) was used according to [41], the mathematical model for relative quantification in real-time PCR. As an internal calibrator, (with a relative expression value of 1.00) the *ces* gene expression at an OD_{600} of 0.2 was chosen. Sample-to-sample variation was corrected by using the 16S rDNA gene as a reference (*rrn*), [54]. Mean values and standard deviations were calculated from three independent experiments and two independent measurements. Statistically significant differences ($p < 0.5$, $p < 0.01$ and $p < 0.001$) compared to the wild-type reference strain *B. cereus* F4810/72 were calculated with a paired, two-tailed Student's *t*-test.

3.10. Cereulide Quantification by Means of Ultraperformance Liquid Chromatography (UPLC) Tandem Mass Spectrometry (MS/MS)

Samples of 5 mL were taken from bacterial cultures at a specific OD_{600} of 4, 7, 10, 14, 16 as well as 24 h and 48 h and centrifuged at 8000× *g*, 2 min, 4 °C. Pellets were stored at −80 °C until further processing. For cereulide extraction, about 50 mg of bacterial mass was weighted by pipetting and re-suspended in the respective amount of ethanol absolute. The cereulide extraction was performed as previously described [42].

The mass spectrometric analysis was performed using a WatersXevo TQ-S mass spectrometer (Waters, Manchester, UK) combined with an Acquity UPLC i-class core system (Waters, Milford, MA, USA), as described previously [55]. The UPLC-MS/MS system was equipped with a 2.1 × 150 mm, 1.7µm, UPLC CSH C_{18} column (Waters, Manchester, UK). Measurements were executed in the positive electrospray ionization (ESI) mode as described previously [55]. The UPLC Xevo TQ-S system was operated with MassLynxTM 4.1 SCN 813 Software (Waters, Manchester, UK), and analysis and data processing were completed using TargetLynx (Waters, Manchester, UK). By means of the multiple reaction monitoring (MRM) mode, the ammonium adducts of cereulide (m/z 1170.7→qualifier: m/z 172.2, 314.2; quantifier: m/z 357.2) were analyzed for a duration of 25 ms. The analysis of MS-data was performed as described [56]. All samples were measured in two different dilutions as duplicates. Mean values and standard deviations were calculated from three independent experiments.

3.11. Sequence Analysis

For the emetic reference strain *B. cereus* F4810/72, the genomic information was retrieved from the NCBI website (GenBank accession numbers NC_011655.1 and CP001179.1) and used for homology search and analysis of the *pagR* sequence. For the reference

B. anthracis Sterne strain (GenBank accession number CP009540.1) and for the reference *B. anthracis* Pasteur strain (GenBank accession number CP009475.1), the genome information was also retrieved from the NCBI website were used for homologue search and analysis of the *pagR* sequence.

The DNA and amino acid sequences were accessed from the NCBI database. Protein homology search was performed by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and as well as PATRIC (https://patricbrc.org/), accessed on 6 August 2022. Multiple sequence alignments were carried out with CLC Workbench Qiagen Software and SnapGene Software (GSL Biotech, USA). The published annotations of the characteristic protein domains of the crystal structure of PagR of *B. anthracis* was used according to [33]. Phylogenetic tree analyses were performed with the construction method neighbor joining and the distance measurement with the Jukes–Cantor model, including bootstrap analysis via CLC Workbench Qiagen Software.

For sequence homology analysis of proteins, the database Emboss Needle program [57] was used (https://www.ebi.ac.uk/Tools/psa/emboss_needle/), accessed on 9 august 2022, due to an optimal sequence alignment, applying the Needleman-Wunsch algorithm.

Predictive 3D protein models were constructed using the Phyre2 online tool (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), accessed on 29 august 2021, by adding the protein sequence, respectively [37].

4. Conclusions

In summary, our work provides novel insights into the complex regulatory circuits governing the non-ribosomal biosynthesis of the depsipeptide toxin cereulide by the CesNRPS. As shown previously, different levels of regulation are involved in the tight control of cereulide production [24]. The chromosomally encoded global regulator CodY, which senses the physiological status of the cell and orchestrates virulence factor expression in emetic *B. cereus*, as well as the pleiotropic transition state regulator AbrB, plays an important role as repressors of *ces* transcription in early growth phases. Both have been shown to act on the timing of *ces* expression by direct binding to the central promoter *cesP* [22,26]. In addition, CesH, which forms part of the *ces* gene locus, has been shown to influence the *ces* expression, but most likely by an indirect mechanism [27] yet to be explored. Furthermore, the ABC transporter CesCD contributes to cereulide production through its recently described non-canonical function in CesNRPS assembly [21].

With the identification of the PagR homologue PagRBc, which represents the first pCER270-encoded transcription factor described to be involved in *ces* transcription, we add a new piece to this still not entirely completed puzzle of virulence gene regulation in emetic *B. cereus* (Figure 7). Furthermore, PagRBc is the first ArsR/SmtB family member shown to be involved in the regulation of non-ribosomal assembly of cereulide by CesNRPS, highlighting the structural and functional diversity of factors involved in the tight control of *ces* expression. The homologous PagR (PagRBa) in *B. anthracis* has been shown to act in a complex cascade of signaling transduction that orchestrates the expression of virulence factors at the right time and in the right place [10,15,58]. It is therefore expected from our now-expanded understanding that PagRBc plays a similar role in the fine tuning of virulence factor expression in emetic *B. cereus*.

In addition, our work revealed that the newly identified PagR homologues PagR1Bc and PagR1Ba are nearly identical and also show a conserved genetic organization of their gene neighborhood, suggesting that they are functionally and structurally interchangeable. Notably, both genes are located on megaplasmids, which carry the key virulence determinants of emetic *B. cereus* (pCER270) and *B. anthracis* (pXO1 Ba), respectively. Thus, further studies should dissect the function of these PagR-like transcription factors for the pathophysiology of emetic *B. cereus* and *B. anthracis*.

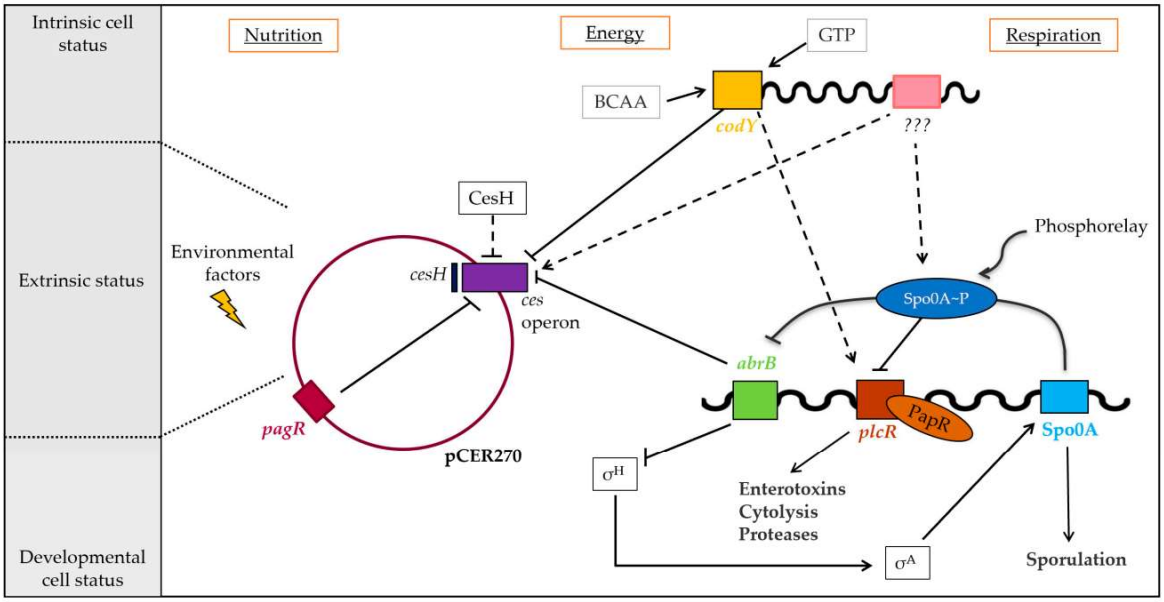

Figure 7. Regulation of cereulide toxin synthesis of emetic *B. cereus*. Cereulide toxin synthesis is a complex and multilayered process, orchestrated by the interplay of chromosomally and plasmid-encoded factors controlling the toxin synthesis at the transcriptional and post-transcriptional levels. The *ces* operon is tightly controlled by the chromosomally encoded global transcriptional regulators CodY and the transition phase regulator AbrB that belongs to the Spo0A regulon, but not by the pleiotropic *B. cereus* virulence regulator PlcR. The putative hydrolase CesH, encoded adjacent to the *ces* operon, indirectly controls cereulide biosynthesis, while the ArsR/SmtB family repressor PagRBc on pCER270 plasmid exerts control of *ces* transcription. Solid arrows: transcriptional regulation; dashed arrows: indirect regulatory effects (adapted from [24]).

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3.2 Manuscript 2

Detection and Isolation of Emetic *Bacillus cereus* Toxin Cereulide by Reversed Phase Chromatography

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Detection and Isolation of Emetic *Bacillus cereus* Toxin Cereulide by Reversed Phase Chromatography

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Abstract: The emetic toxin cereulide is a 1.2 kDa dodecadepsipeptide produced by the food pathogen *Bacillus cereus*. As cereulide poses a serious health risk to humans, sensitive and specific detection, as well as toxin purification and quantification, methods are of utmost importance. Recently, a stable isotope dilution assay tandem mass spectrometry (SIDA–MS/MS)-based method has been described, and an method for the quantitation of cereulide in foods was established by the International Organization for Standardization (ISO). However, although this SIDA–MS/MS method is highly accurate, the sophisticated high-end MS equipment required for such measurements limits the method's suitability for microbiological and molecular research. Thus, we aimed to develop a method for cereulide toxin detection and isolation using equipment commonly available in microbiological and biochemical research laboratories. Reproducible detection and relative quantification of cereulide was achieved, employing reversed phase chromatography (RPC). Chromatographic signals were cross validated by ultraperformance liquid chromatography–mass spectrometry (UPLC–MS/MS). The specificity of the RPC method was tested using a test panel of strains that included non-emetic representatives of the *B. cereus* group, emetic *B. cereus* strains, and cereulide-deficient isogenic mutants. In summary, the new method represents a robust, economical, and easily accessible research tool that complements existing diagnostics for the detection and quantification of cereulide.

Keywords: cereulide; reversed phase chromatography (RPC); Äkta™ pure; peptide quantification; emetic *Bacillus cereus*; toxin purification

Key Contribution: Analysis of cereulide of emetic *B. cereus* was achieved using reversed phase chromatography (RPC), and cross-validation was performed by ultraperformance liquid chromatography-mass spectrometry (UPLC–MS/MS). This new method represents a robust; economical; and easily accessible tool for cereulide detection and relative quantification

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1. Introduction

The emetic toxin, cereulide, is a dodecadepsipeptide, composed of six α-amino acid, and six α-hydroxy acid, moieties arranged in three repeating tetradepsipeptide units $[D\text{-}O\text{-}Leu\text{-}D\text{-}Ala\text{-}L\text{-}O\text{-}Val\text{-}L\text{-}Val]_3$ [1]. Cereulide is produced by an emetic subgroup of *Bacillus cereus*, a bacterial pathogen typically associated with food poisoning [2,3]. Similarly to other highly bioactive peptides, such as the antibiotic valinomycin produced by *Streptomyces* spp., cereulide is synthesized by a non-ribosomal peptide synthetase (NRPS), designated CesNRPS [4–6]. The *cesNRPS* genes are organized as an operon within a 24 kb multigene cluster located on the pCER270 megaplasmid, which shares its backbone with

the pXO1 toxin plasmid of *Bacillus anthracis* [7]. The structural *cesAB* genes, which play a pivotal role in cereulide biosynthesis, are co-transcribed as a single polycistronic mRNA with adjacent genes [8–10]. Cereulide accumulates during growth of emetic *B. cereus* in a growth temperature range from 12 °C to 40 °C [11,12], and reaches high levels in the stationary phase [8,13,14]. However, cereulide production capability among emetic *B. cereus* strains varies up to 1000-fold [4,15,16].

Due to its cyclic structure, cereulide is extremely resistant against heat, extreme pH conditions, as well as proteolysis [17], and cannot be inactivated by standard hygienic measures in food production and processing. Furthermore, cereulide is not inactivated during stomach passage in the host, because of the peptide's resistance to cleavage by pepsin and trypsin [18–20]. Thus, cereulide represents a serious challenge for the food industry, as severe intoxications linked to cereulide are on the rise [12,21]. Key symptoms of cereulide intoxication are fulminant episodes of vomiting shortly after the consumption of cereulide contaminated food, nausea, and abdominal cramps [14]. Due to its hepatotoxic activity, cereulide can cause liver damage, rhabdomyolysis, and severe multi-organ failure [22–25]. Documented biological activities of cereulide include emesis in primates [18,26], and swelling of mitochondria in HEp-2-cells [27]. Cereulide has also been linked to the induction of diabetes by causing beta cell dysfunctions [28]. In addition, neurological symptoms, such as seizures and lethargy, similar to those described from human intoxications have been reported in intoxication studies using a porcine model [29]. The intoxication studies also showed that cereulide can accumulate in several organs and tissues, and possibly cross the blood–brain barrier [29].

Due to the lack of fast and specific cereulide quantification and isolation methods, purification and quantification of the toxin is still laborious. Nevertheless, considerable progress has been made in the diagnostics of emetic *B. cereus* [30], and methods for specific detection of emetic *B. cereus* strains by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-ToF) mass spectrometry (MS) have been published recently [31,32]. Since MALDI-ToF MS is increasingly used in routine microbiology laboratories, it is expected that these methods, allowing the discrimination of emetic and non-emetic *B. cereus*, will significantly improve differential *B. cereus* diagnostics in clinical settings, as well as in foodborne outbreak situations. A drawback of the current MALDI-ToF MS methods are the mass spectrometers commonly used in routine diagnostics; which do not allow accurate quantification of cereulide [31]. Thus, liquid chromatography coupled to mass spectrometry (LC-MS) is still considered the gold standard for cereulide detection and quantification [33–36]. Based on stable isotope mass spectrometry-based dilution assay (MS-SIDA) [33], an EN-ISO method (EN ISO 18465) for quantitation of cereulide in food matrices was recently established [37].

However, although the latter MS method is characterized by high accuracy, its use in microbiological research is limited by the financial burden of the MS equipment required. We therefore aimed to develop a method suitable for research in microbiological and biochemical laboratories, using equipment commonly available there, such as reversed phase chromatography (RPC) systems for the purification of proteins, peptides, and nucleic acids. These flexible chromatography systems allow for quick, simple, and easy customization. Owing to their broad applicability and the low costs (compared to sophisticated UPLC-MS/MS), RPC systems are frequently available in microbiological and biochemical laboratories. Here, we employed an Äkta™ pure RPC chromatography system, to assess its suitability for cereulide research. Our work suggests that RPC systems are indeed valuable tools for cereulide toxin research, allowing, not only its detection, but also the purification, isolation, and relative quantification of cereulide.

2. Results and Discussion

2.1. Establishing a Work Flow for Cereulide Detection from Emetic B. cereus by RPC: Cultivation of Bacteria and Crude Cereulide Extraction (Step 1–2)

A set of strains for cereulide production, detection, and purification by RPC was compiled, including emetic *B. cereus* strains with different cereulide biosynthesis capacities,

isogenic mutant strains, as well as non-emetic representatives of the *B. cereus* group. A list of strains included in this panel is provided in Table 1.

Table 1. *Bacillus cereus* group strains used for method establishment of cereulide purification and quantification by reversed phase chromatography (RPC).

Strain-ID	Relevant Genotype and Characteristics	References
ATCC 14579	Non-emetic *Bacillus cereus* type strain	[38]
F4810/72	Emetic *B. cereus* reference strain, also termed AH187, isolated from vomit; emetic food-borne outbreak in UK	[3,26]
ATCC 10792	*Bacillus thuringiensis* type strain	[39]
RIVM BC90 [a]	*B. cereus* isolated from human faces; diarrheal outbreak in The Netherlands	[3,26]
F48Δ*cesP/polar* [b]	F4810/72 Δ*cesP*::*spc*, Spc^r; cereulide deficient due to transcriptional inactivation of *cesABCD* genes	[9]
F48Δ*abrB* [c]	F4810/72 Δ*abrB*::*spc*, Spc^r; cereulide overproduction due to deletion of transcription regulator *abrB*	[40]
F48Δ*pBCE* [b]	F4810/72 Δ*pBCE270*; cereulide deficient due to deletion of *ces* locus encoding plasmid pBCE270	[Dommel and Ehling-Schulz; unpublished]
F5881/94	Emetic toxin producing *B. cereus* strain isolated from Chinese takeaway fried rice; emetic food-borne outbreak in UK	[3,26]
RIVM BC379	Emetic toxin producing *B. cereus* isolated from chicken; The Netherlands	[34]
Bacillus anthracis Sterne	Attenuated vaccine strain, which lacks virulence plasmid pXO2	[41]

[a] emetic-like strain as defined by Ehling-Schulz et al. [3]; [b] cereulide deficient mutant; [c] cereulide overproducing mutant.

Strains were grown under standardized laboratory conditions in LB medium (Lysogeny broth medium) at 30 °C and rotary shaking at 120 rpm for 24 h, using a previously established protocol for kinetic inoculation to ensure reproducibility [5]. Cells were harvested by centrifugation, and subsequently cereulide was extracted from the cells at room temperature with ethanol overnight. Next, cells were pelleted by centrifugation and supernatants were collected. After removal of cell debris from the supernatants by filtration (0.2 µm filter size), the crude cereulide extract was either subjected directly to UPLC-MS/MS for quantitation (see Section 2.3), or the cereulide extract was processed for analysis and further cereulide isolation by RPC (see Section 2.2). A schematic overview of the complete workflow is depicted in Figure 1.

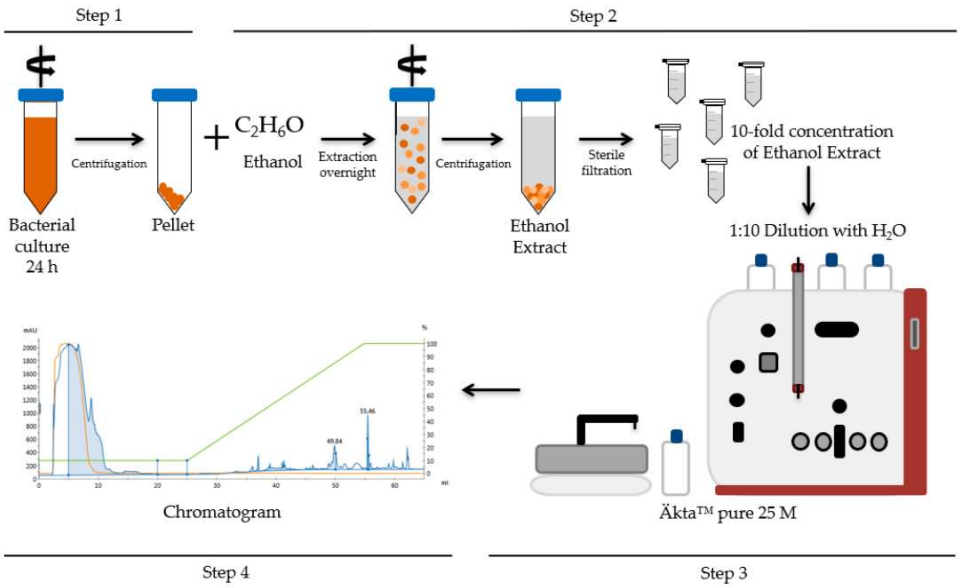

Figure 1. Schematic principle of cereulide analysis by reversed phase chromatography (RPC). Step 1: Cultivation of bacterial strains; Step 2: Extraction procedure; Step 3 + 4: Cereulide toxin isolation and quantification by RPC using an Äkta™ pure system and a silica based C12 column.

2.2. Identification and Isolation of Purified Cereulide Toxin (Step 3-4): Cereulide Chromatogram on an Äkta™ Pure 25M Using a Silica Based RP C12 Column

Due to the highly hydrophobic character of cereulide [42], RPC was performed using a silica based C12 column and an Äkta™ pure 25 M instrument to separate hydrophobic substances from ethanol extracts of *B. cereus* group strains, and to identify cereulide. For separation of the metabolites in the ethanol extracts a linear gradient from 10% ethanol to ethanol absolute for 60 min, followed by 15 min at ethanol absolute was used. UV absorption at 280 nm (proteins) and 210 nm (peptides) was simultaneously recorded. A specific chromatographic signal during RPC at 210 nm after 55.5 mL was detected in the emetic reference strain F4810/72, while this peak was absent in all cereulide negative *B. cereus* group strains, such as the *B. cereus* type strain, the *Bacillus thuringiensis* type strain, and *Bacillus anthracis* (Figure 2). The fraction corresponding to the specific peak at 55.5 mL in the emetic reference strain F4810/72 was subjected to UPLC–MS/MS analysis and identified as cereulide (for details see Section 2.3).

Furthermore, this cereulide specific chromatographic signal was absent in the emetic like strain, RIVM BC90. Emetic like strains are strains that are genetically closely related to emetic strains, and share certain phenotypic characteristics with the latter, except that they do not possess *ces* genes and are thus unable to produce cereulide [2,3]. Based on the close relatedness, it could be expected that the metabolite pattern in the ethanol extracts of emetic like strains might be similar to the ones of emetic strains, except for the absence of cereulide. Similarly, it could be expected that the isogenic cereulide-deficient mutant F48Δ*cesP/polar* of the emetic reference strain F4810/72 would share, excluding its cereulide deficiency, general phenotypic features with its parental strain, reflected in the ethanol extracts of metabolites. As shown in Figure 2, the metabolite pattern in the ethanol extracts of emetic-like strain RIVM BC90 and the cereulide-deficient mutant, F48Δ*cesP/polar* were indeed more similar to the pattern of the emetic strain F4810/72, than to those of the more distantly related non-emetic *B. cereus* group strains. Yet, similarly to the chromatograms from the non-emetic *B. cereus* group strains, the specific peak detected in F4810/72 was

absent from their respective chromatograms, fostering the hypothesis that the peak at 55.5 mL is specific to cereulide producing emetic *B. cereus*.

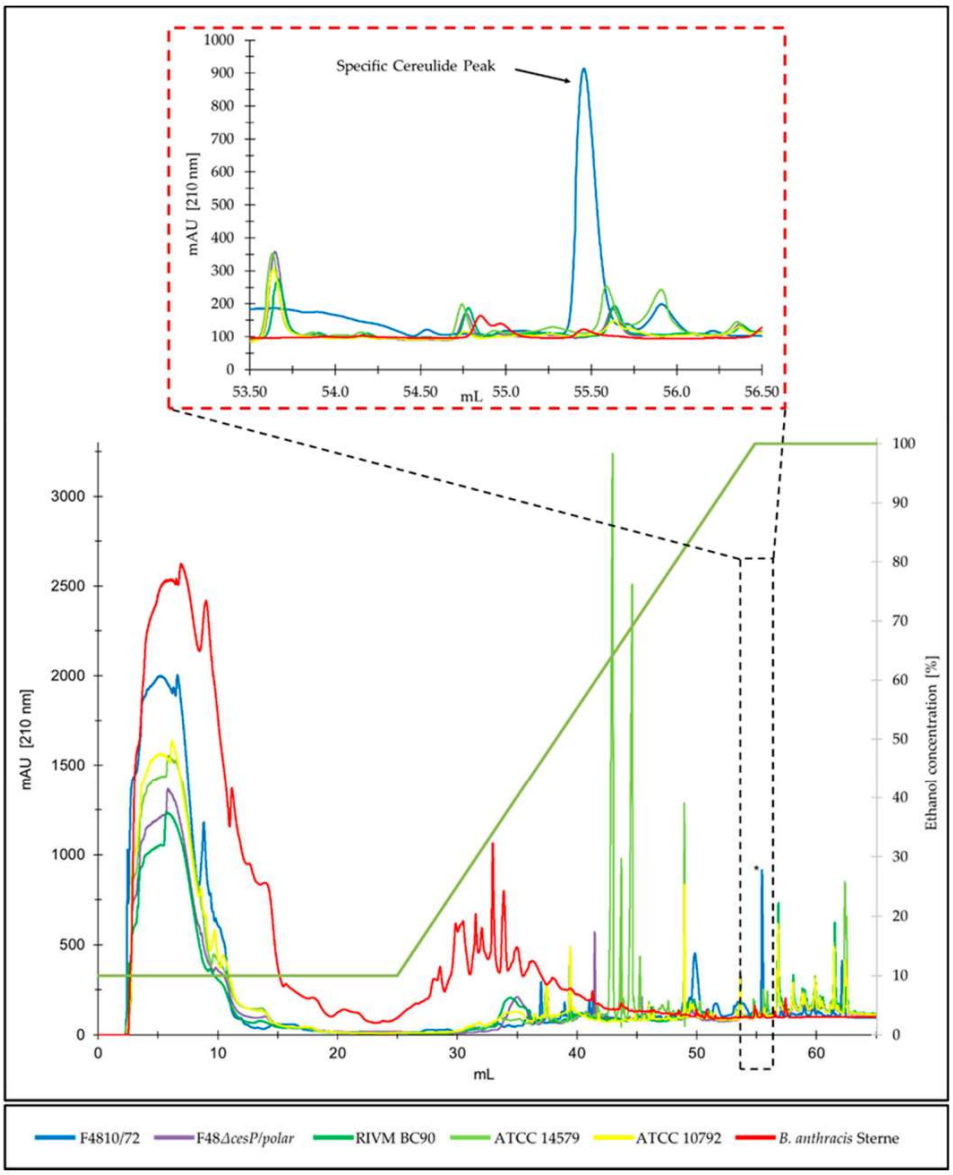

Figure 2. Reversed phase chromatogram of ethanol extracts from the emetic reference strain F4810/72 (blue) and the cereulide negative isogenic mutant F48Δ*cesP/polar* (purple), as well as of selected non-cereulide producing *B. cereus* group strains: emetic-like *B. cereus* RIVM B90 (green), *B. cereus* type strain ATCC 14579 (light green), *B. thuringiensis* type strain ATCC 10792 (yellow), and *B. anthracis* Sterne (red). Strains were grown in LB (Lysogeny broth) for 24 h at 30 °C, cereulide was extracted from cells with ethanol absolute over-night, and concentrated 10-fold. Ethanol extracts were diluted 1:10 in water (*v*/*v*) and analyzed by RPC, as described in the material and method section. The specific cereulide peak at 55.5 mL (see inset) was collected using automatic peak sampling, and subsequently quantified by ultraperformance liquid chromatography–mass spectrometry (UPLC-MS/MS). A representative result from three independent experiments is shown.

To test this hypothesis, we next analyzed two additional emetic *B. cereus* strains, one high (F5881/94), and one low, cereulide producer (RIVM BC379) [34]. As depicted in Figure 3, extracts of both strains showed the prominent peak at 55.5 mL, highlighting the specificity of this peak. Notably, the high cereulide producer strain F5881/94 exhibited a higher peak than the medium toxin producer strain F4801/72, while the peak of the low cereulide producer (RIVM BC379) was even lower than the peak of F4801/72, indicating that our method may be used for relative quantification and classification of emetic strains (see Table 2).

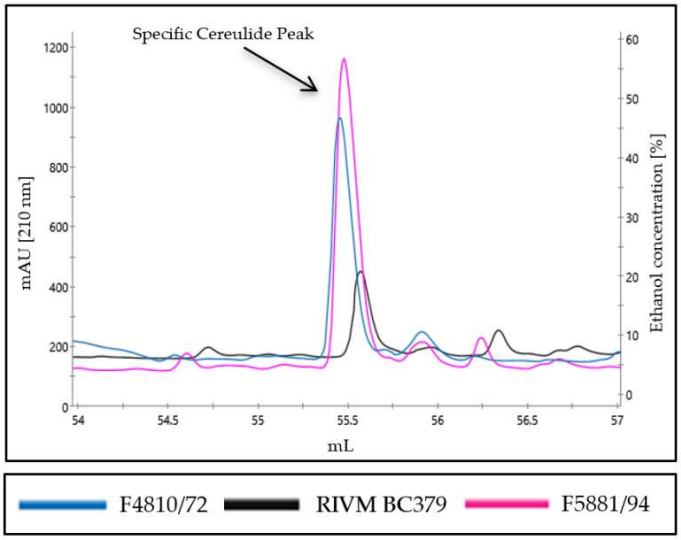

Figure 3. Reversed phase chromatogram of ethanol extract from emetic *B. cereus* strains with different cereulide production capacities, such as the medium toxin producer F4810/72 (blue), the high toxin producer F5881/94 (pink), and the low toxin producer RIVM BC379 (black). Strains were grown in LB broth, processed, and analyzed by RPC, as described in the material and method section. A representative result from three independent experiments is shown.

Next, we tested the suitability of our new RPC method for a rapid analysis of the cereulide production capacities of mutant strains, as this allowed for a relative quantification of cereulide. To this end, two cereulide deficient mutant strains, one cereulide overproducing mutant and the parental strain F4810/72 were grown in LB broth, with rotary shaking at 120 rpm, for 24 h, at 30 °C and at 37 °C. Samples were processed as described above. As expected, RPC of the respective ethanol extract from the mutant strains revealed that the peak at 55.5 mL was absent in the cereulide-deficient mutants F48Δ*cesP/polar* and F48Δ*pBCE*, while it was higher in the cereulide-overproducing mutant F48Δ*abrB* compared to the parental F4810/72 (Figure 4), reflecting the previously reported upregulated cereulide production in this mutant strain [40].

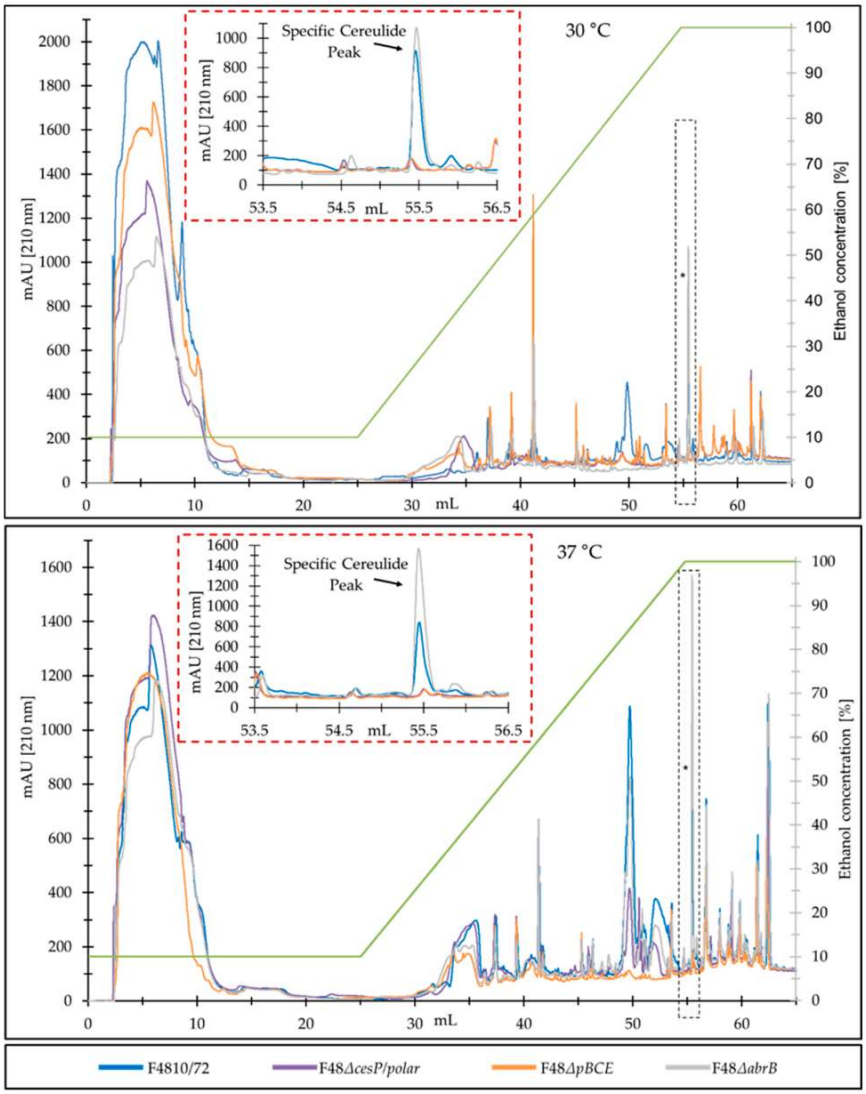

Figure 4. Reversed phase chromatogram of ethanol extracts from wildtype F4810/72 and its isogenic mutants. The specific cereulide peak at 55.5 mL from wildtype F4810/72 is indicated by an asterisk. The strains were grown in LB broth for 24 h at 30 °C or 37 °C, respectively, extracted with ethanol absolute over-night and concentrated 10-fold. Ethanol extracts were diluted 1:10 in water (v/v) and analyzed by RPC. The specific cereulide peak at 55.5 mL was collected and quantified by UPLC–MS/MS. A representative result from three independent experiments is shown.

As shown in Figure 4, cereulide production in the *abrB* deletion mutant was increased even more at 37 °C than at 30 °C compared to the parental strain. The temperature effect on cereulide production in the F48Δ*abrB* detected by our RPC method warrants further investigation, as AbrB is an important, pleiotropic transition phase regulator in Bacilli, which is still far from being fully understood.

The respective RPC fractions that eluted after 55.5 ± 0.1 mL, and showed the cereulide specific signal in the ethanol extracts of the emetic strains, were collected from all strains included in the study and subjected to UPLC-MS/MS [33] to confirm the specificity of the corresponding chromatographic signal for cereulide (see Section 2.3).

In addition, the biological activity of the purified toxin cereulide in the respective positive RPC fractions of the emetic reference strain F4810/72, was tested using human larynx carcinoma (HEp-2) cells, as described previously [4]. The HEp-2-cell assay confirmed the biological activity of cereulide in the respective RPC fraction. Thus, the RPC method described here may be useful, not only for detection of cereulide, but also for the isolation, purification, and concentration of cereulide to be used in further functional in vitro and *in vivo* studies, to fully decipher the mode of action of this toxin.

Table 2. Quantification of cereulide by means of UPLC-MS/MS before/after cereulide purification/enrichment by RPC, and comparison of cereulide-specific peak areas at 55.5 mL. Part of the ethanol extracts from each strain was subjected directly to LC-MS/MS analysis, while parts of the extracts were processed via RPC. The respective RPC fraction eluting after 55.5 ± 0.1 mL was subsequently analyzed by UPLC-MS/MS. The cereulide-specific peak areas at 55.5 mL were quantified for comparison of the relative amounts of UPLC-MS/MS- and RPC-enriched cereulide fractions. Abbreviations: N.D.: not detected. Std. Dev.: standard deviation [2]. mAU: milli-absorbance unit.

	Ethanol Extracts of *B. cereus* Directly Subjected to UPLC-MS/MS Analysis	**RPC-Enriched Cereulide Fractions (55.5 mL) Subjected to UPLC-MS/MS**	**Cereulide-Specific Peak Area of RPC at 55.5 mL**
Strain-ID [1]	**Cereulide [µg/mL] ± Std. Dev. [2]**	**Cereulide [µg/mL] ± Std. Dev. [2]**	**mL*mAU ± Std. Dev. [2]**
F4810/72	3.8 ± 2.7	33.4 ± 10.0	91.6 ± 28.4
F48Δ*abrB*	10.4 ± 1.3	82.9 ± 18.1	113.8 ± 27.0
F5881/94	17.9 ± 7.2	95.7 ± 12. 9	127.8 ± 26.4
RIVM BC379	2.1 ± 1.3	24.4 ± 6.4	23.0 ± 10.7
ATCC 14579	N.D.	N.D.	N.D.
RIVM BC90	N.D.	N.D.	N.D.
F48Δ*cesP*/polar	N.D.	N.D.	N.D.
F48Δ*pBCE*	N.D.	N.D.	N.D.
ATCC 10792	N.D.	N.D.	N.D.
B. anthracis Sterne	N.D.	N.D.	N.D.

[1] Emetic strains producing cereulide are indicated in bold. [2] Means and standard deviations are derived from $n = 3$ independent experiments (for details see Section 4).

2.3. Method Validation by UPLC-MS/MS-Analysis

The described method for cereulide identification and purification by RPC was cross-validated by means of UPLC-MS/MS. For this purpose, ethanol extracts from each strain of the strain panel were analyzed in parallel by UPLC-MS/MS before and after RPC purification of cereulide. As shown in Table 2, the UPLC-MS/MS analyses confirmed that the peak at 210 nm, after 55.5 mL in RPC, which was uniquely detected in cereulide-producing emetic *B. cereus* strains, indeed contained cereulide. A comparison of the peak areas of cereulide specific peaks in RPC for the different strains and amounts of cereulide determined by UPLC-MS/MS revealed a correlation, which indicates that our RPC method presents a suitable tool for the relative quantification of cereulide and the classification of emetic strains (see Table 2).

All ethanol fractions from emetic strains showing a presumably specific cereulide signal in RPC, were tested positive in the UPLC-MS/MS. Even detection and isolation of cereulide from the low-level emetic *B. cereus* strain RIVM BC379 was possible by RPC. Conversely, no cereulide was detected by UPLC-MS/MS in any of the RPC fractions obtained from the non-emetic *B. cereus* group strains or cereulide-deficient isogenic mutants of the emetic reference strain F4810/72 cereulide, confirming the specificity and sensitivity of the RPC method. Notably, compared to direct analysis of the ethanol extracts by UPLC-MS/MS, the RPC purification protocol resulted in significant enrichment of

cereulide (Table 2). Although synthetically produced cereulide has become available in recent years [35], bio-fermentative production of cereulide and subsequent purification using our new RPC method might present a more economical alternative.

Since RPC has been successfully employed to screen for cyanobacterial peptide toxins in water samples [43], it could be assumed that our method might be suitable for screening of cereulide in water or other environmental samples. Although there is increasing evidence that various water reservoirs and water cycles may be important sources of *B. cereus* contamination [44–46] and a highly toxic emetic strain isolated from a drinking bottle has been associated with severe cereulide intoxication [34], literally nothing is known about the occurrence of cereulide in water. Thus, our novel RPC method could provide a suitable tool for systematical surveys of water and sediments for cereulide contamination, to decipher the actual contribution of these unexplored niches to cereulide mediated intoxications.

However, it should also be mentioned that the RPC method described here cannot be used for direct detection of cereulide in complex matrices, such as foods, as these complex matrices may interfere with the current approach. Furthermore, in contrast to the SIDA LC-MS/MS for quantitation of cereulide [33], the new RPC does not allow for absolute quantitation of cereulide. Due to the limited resolution of RPC, isotope labelled cereulide, which has been previously shown to be an ideal internal standard for cereulide quantification by UPLC-MS/MS [33], cannot be included in our method. Although valinomycin has been used as surrogate for cereulide in bioassays [5,40], several studies showed that it is not a suitable standard for quantification of cereulide by analytical chemical methods [33,35]. Thus, we have refrained from including it as an internal standard in our RPC method.

Nevertheless, our results from the UPLC-MS/MS analysis of RPC fractions revealed that RPC might be suitable for the relative quantitation of cereulide. For instance, it could be used to classify strains as high- or low-level cereulide producers relative to the emetic reference strain F4810/72, or to test the cereulide production capacity of a mutant strain compared to its parental strain (see Figures 3 and 4). Thus, the RPC method established in this study could become a valuable tool for research, and complementary to UPLC–MS/MS for accurate quantitation of cereulide in outbreak situations and MALDI-ToF MS for rapid detection of emetic *B. cereus* strains in the frame of routine microbial diagnostics.

3. Conclusions

In conclusion, RPC was successfully applied for the detection, relative quantification, and isolation of cereulide, which was validated by comparing results with canonical UPLC-MS/MS. Since many microbiological and biochemical research laboratories are equipped with systems for RPC, we expect that our new method can become an economical and easy to implement tool that complements existing more elaborate diagnostic tools for cereulide detection and quantification. In addition, by adjusting the sample preparation protocol and optimizing chromatographic conditions, including adaptations of detector settings and running conditions, the RPC method presented here could also become a suitable tool for cereulide detection in more complex specimens, such as foods, in the future.

4. Materials and Methods

4.1. Test Set of B. cereus Group Strains

A test set of *B. cereus sensu lato* group strains (n = 10) was compiled and used to test the suitability of RPC for cereulide analysis (Table 1). The strain panel included three emetic *B. cereus* strains, one emetic-like strain that shares several physiological features with emetic strains, but is not able to produce cereulide as it lacks the *ces* genes [2,3], as well as three non-emetic *B. cereus* group strains, and three isogenic mutants of the emetic *B. cereus* strain F4810/72, which are either cereulide-deficient or biosynthesize cereulide at different levels.

4.2. Cultivation of Bacterial Strains (Step 1)

All bacterial strains were cultivated on LB-Miller (LB) agar plates (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) and incubated overnight at 30 °C. Liquid bacterial cultures were inoculated in 3 mL of LB broth, and incubated at 30 °C for 16 to 18 h at 120 rpm. Fresh cultures were inoculated at a final inoculum of 10^3 CFU/mL in 100 mL of LB broth in bottom-baffled 500-mL-flasks, and incubated at 120 rpm for 24 h at 30 °C, as described previously [8]. Cells were harvested by centrifugation at 8600× *g* for 12 min at room temperature, and the supernatant was discarded. The pellets were frozen in liquid nitrogen and stored at −80 °C until use. Three biological replicates, before and after purification by RPC, were quantified by UPLC-MS/MS analysis.

4.3. Cereulide Extraction Procedure (Step 2)

The following extraction procedure was carried out for each culture: After thawing the pellets on ice, 1 g (wet weight) of cell material was transferred to a 50 mL tube. Then, 10 mL of ethanol absolute was added, and the pellet was resuspended gently by shaking or pipetting. Extraction was performed overnight at room temperature on a rocking table. The lid was covered with parafilm to avoid leakage, and was additionally covered with aluminum foil to protect the sample from light.

On the next day, the suspension was centrifuged for 12 min at 8600× g at room temperature and filtered through a 0.2 µm PTFE filter (polytetrafluorethylen; Phenomenex, Aschaffenburg, Germany). The remaining extract was aliquoted to 2 mL and stored at −20 °C until use. The extracts were concentrated 10-fold using a concentrator with an integrated vacuum pump (Eppendorf, Hamburg, Germany) at 45 °C for about 1 h, and extracts from the same strain were pooled to a final volume of 1 mL.

4.4. Cereulide Toxin Purification by RPC, (Step 3–4)

The ethanol extract of 1 mL was diluted 1:10 (v/v) in double distilled water. The suspension was gently mixed by inverting the tube 5 times, or until a homogeneously colored suspension was achieved. For RPC, an Äkta™ pure 25 M system with a fraction collector F9-C (GE Healthcare, Solingen, Germany) was employed, and a 5 mL sample loop (PEEK, polyetheretherketone; GE Healthcare, Solingen, Germany) was used for application of the diluted sample extracts.

Due to the highly hydrophobic character of cereulide, the purification of cereulide was performed using a silica based C12 column (Jupiter® 4 µm Proteo 90 Å, LC Column, size 250 * 4.6 mm; Phenomenex, Aschaffenburg, Germany) as RPC, and a pre-column SecurityGuard Cartridge Kit Kj0-4282 (Phenomenex, Aschaffenburg, Germany) with security cartridges filters (Phenomenex, Aschaffenburg, Germany) for the pre-column as the fast protein liquid chromatography (FPLC) system. All solutions were degassed in an ultrasonic bath before use.

UV absorption at 280 nm and 210 nm was simultaneously measured with a UV detector (UV monitor (U9-L), (GE Healthcare, Solingen, Germany). The method-based unit was column volumes (CV), and the default flow rate was set to 0.5 mL/min (control flow to avoid overpressure). PEEK Tubing from the injection valve, column valve, column, UV monitor, and conductivity monitor was changed to PEEK Tubing of ID 0.25 mm OD 1/16" (GE Healthcare, Solingen, Germany) to build-up a higher pressure for cereulide toxin purification.

The running conditions for toxin quantifications were inlet A1 for MQH_2O, Inlet B1 for ethanol absolute, and inlet B2 for 65% acetonitrile. In this method, all percentage values of ethanol and acetonitrile refer to percentage of volume (% vol). The whole program for cereulide toxin purification and quantification (see Supplementary File S1) included the following steps:

1. Preparation step: the flow rate was set to 0.5 mL/min, and the column was washed with 1 CV of 65% acetonitrile. A linear gradient was performed from 65% to 6.5% of acetonitrile within 2 CV.

2. Equilibration step: the column was equilibrated with 4 CV of 10% ethanol.
3. Sample application: the sample was applied directly to the column using a prefilled 5 mL capillary loop (GE Healthcare, Solingen, Germany).
4. Washing step: an equilibration buffer was used to remove all unbound hydrophilic substances. Fractions of unbound protein were collected using the fraction collector F9-C.
5. Elution step: to elute bound molecules, a 11.50 CV linear gradient of 10% ethanol to ethanol absolute in running buffer (MQH_2O) was applied. Subsequently, the column was washed with ethanol absolute for 5 CV in running buffer (MQH_2O). A linear gradient from ethanol absolute to 10% ethanol in running buffer (MQH_2O) within 3 CV was performed, and the column was washed with 1 CV of 10% ethanol in running buffer (MQH_2O). Automatic peak fractionation was used to collect fractions >200 mAU (milli-absorbance unit). Cereulide eluting from the column was detected in the 55.5 ± 0.1 mL fraction at a wavelength of 210 nm. Fractions of eluted cereulide were transferred to screw neck vials N9 (1.5 mL, 11.6 × 32 mm with N9 PP screw caps with red rubber; Machery–Nagel, Düren, Germany) for UPLC-MS/MS analysis.
6. Follow-up step 1: the column was washed with 10% ethanol in running buffer (MQH_2O) for 2 CV.
7. Follow-up step 2: a linear gradient from 10% ethanol to ethanol absolute in running buffer (MQH_2O) within 1.5 CV was performed.
8. Follow-up step 3: the column was washed with 2 CV ethanol absolute.
9. Equilibration step No. 1: the column was equilibrated with 95% acetonitrile for 4 CV.
10. Equilibration step No. 2: the column was equilibrated with 61.75% acetonitrile for 4 CV.

4.5. Method Validation by Ultraperformance Liquid Chromatography-Mass Spectrometry (UPLC-MS/MS)

To confirm the identity of cereulide in the 55.5 mL fraction derived from RPC analysis (see Section 4.3), these fractions were analyzed by UPLC-MS/MS together with the ethanol extracts from the *B. cereus* group strains, which had not been subjected to RPC beforehand.

The mass spectrometric analysis was performed according to the literature on a Waters Xevo TQ-S mass spectrometer (Waters, Manchester, UK) combined with an Acquity UPLC i-class core system (Waters, Milford, MA, USA), comprising a binary solvent manager, sample manager, and column oven [36]. Aliquots (2 µL) of the prepared samples were injected into the UPLC-MS/MS system equipped with a 2.1 × 150 mm, 1.7 µm, UPLC CSH C18 column (Waters, Manchester, UK). The UPLC unit was operated at a flow rate of 0.7 mL/min and a temperature of 55 °C, applying the following gradient with $HCOONH_4$ (10 mmol, 0.1% HCOOH) as solvent A, and MeCN (0.25% HCOOH) as solvent B. Chromatography was started at 85% B, increased to 95% B within 8.0 min, increased to 99% B within 0.1 min, kept constant for 0.9 min, decreased to 85% B within 0.1 min, and followed by re-equilibration at 85% B for 0.9 min. Measurements were executed in the positive electrospray ionization (ESI) mode, with quantitative calibration mode consisting of the following ion source parameters: capillary voltage +3.6 kV, sampling cone 50 V, source offset 35 V, source temperature 150 °C, desolvation temperature 650 °C, cone gas 250 L/h, desolvation gas 1100 L/h, collision gas flow 0.15 mL/min, and nebulizer gas flow 7.0 bar. The mass spectrometer was calibrated using a solution of phosphoric acid (0.1% in MeCN) in the range from m/z 40–1963. The UPLC Xevo TQ-S system was operated with MassLynxTM 4.1 SCN 813 Software (Waters, Manchester, UK), and analysis and data processing were completed using TargetLynx (Waters, Manchester, UK). By means of the multiple reaction monitoring (MRM) mode, the ammonium adducts of cereulide (m/z 1170.7 → qualifier: m/z 172.2, 314.2; quantifier: m/z 357.2), and $^{13}C_6$-cereulide (m/z 1176.7 → m/z qualifier: 173.2, 316.2; quantifier: m/z 358.2) were analyzed for a duration of 25 ms, observing the mass transitions. ESI + mass and product ion spectra were acquired with direct flow infusion using IntelliStart. The MS/MS parameters were tuned for each individual compound, detecting the fragmentation of the $[M + NH_4]^+$ molecular ions into specific product ions after collision with argon. All samples were measured in two different dilutions as duplicates. Mean values and standard

deviations were calculated from three independent experiments. A detailed protocol of the RPC for the purification of cereulide is provided in the Supplementary File S1.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6651/13/2/115/s1, File S1: Full Method Protocol.

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3.3 Manuscript 3

Enterotoxin production of *Bacillus thuringiensis* isolates from biopesticides, foods, and outbreaks

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Enterotoxin Production of *Bacillus thuringiensis* Isolates From Biopesticides, Foods, and Outbreaks

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While the relevance of *Bacillus (B.) cereus* as a major cause of gastroenteritis is undisputed, the role of the closely related *B. thuringiensis* in foodborne disease is unclear. *B. thuringiensis* strains frequently harbor enterotoxin genes. However, the organism has only very rarely been associated with foodborne outbreaks, possibly due to the fact that during outbreak investigations, *B. cereus* is routinely not differentiated from *B. thuringiensis*. A recent EFSA scientific opinion stresses the urgent need for further data allowing for improved risk assessment, in particular as *B. thuringiensis* is a commonly used biopesticide. Therefore, the aim of this study was to gain further insights into the hazardous potential of *B. thuringiensis*. To this end, 39 *B. thuringiensis* isolates obtained from commercially used biopesticides, various food sources, as well as from foodborne outbreaks were characterized by *panC* typing, *panC*-based SplitsTree analysis, toxin gene profiling, FTIR spectroscopic analysis, a cytotoxicity assay screening for enterotoxic activity, and a sphingomyelinase assay. The majority of the tested *B. thuringiensis* isolates exhibited low (23%, $n = 9$) or mid level enterotoxicity (74%, $n = 29$), and produced either no (59%, $n = 23$) or low levels (33%, $n = 13$) of sphingomyelinase, which is reported to act synergistically with enterotoxins Nhe and Hbl. One strain isolated from rosemary was however classified as highly enterotoxic surpassing the cytotoxic activity of the high-level reference strain by a factor of 1.5. This strain also produced vast amounts of sphingomyelinase. Combining all results obtained in this study into a fingerprint pattern, several enterotoxic biopesticide strains were indistinguishable from those of isolates from foods or collected in association with outbreaks. Our study shows that many *B. thuringiensis* biopesticide strains exhibit mid-level cytotoxicity in a Vero cell assay and that some of these strains cannot be differentiated from isolates collected from foods or in association with outbreaks. Thus, we demonstrate that the use of *B. thuringiensis* strains as biopesticides can represent a food safety risk, underpinning the importance of assessing the hazardous potential of each strain and formulation used.

Keywords: *Bacillus thuringiensis*, *Bacillus cereus* group, enterotoxicity, Vero cell assay, sphingomyelinase

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INTRODUCTION

The *Bacillus cereus sensu lato* group comprises *B. cereus sensu strictu* and multiple other closely related species, including *B. thuringiensis* (Schnepf et al., 1998; Ehling-Schulz et al., 2011). *B. thuringiensis* and *B. cereus s. s.* are genetically intermingled and can only be differentiated by the presence or absence of insecticidal toxins, which are delineating the species *B. thuringiensis* (Schnepf et al., 1998; Ehling-Schulz et al., 2011). Since these two species are indistinguishable using cultural detection methods or 16S rDNA sequencing (Ehling-Schulz and Messelhäusser, 2013), they have been suggested to represent the same species (Helgason et al., 2000).

B. cereus s. s. causes two distinct forms of gastrointestinal disease—the diarrheal and the emetic type of *B. cereus* gastroenteritis. The emetic syndrome is caused by oral intake of the emetic toxin cereulide, which elicits nausea and vomiting and is mostly associated with cooked rice dishes (McKillip, 2000; Ehling-Schulz et al., 2004). By contrast, the diarrheal syndrome is caused by the heat-labile enterotoxins Nhe, Hbl, and CytK (Stenfors Arnesen et al., 2008) and is often associated with contaminated meats, sauces, and dairy products (McKillip, 2000). Sphingomyelinase (SMase), a virulence factor structurally related to *Staphylococcus aureus* beta toxin, has been reported to interact synergistically with Nhe and Hbl. SMase has been shown to enhance Hbl hemolysis (Beecher and Wong, 2000) as well as *in vitro* cytotoxicity (Doll et al., 2013). Furthermore, strains producing high levels of SMase that had been isolated from patients with sepsis and endophthalmitis were found to be lethal in mice (Oda et al., 2012), suggesting that the contribution of SMase to *B. cereus* virulence may have been underestimated. Results from *in vivo* studies using *B. cereus* mutants foster the hypothesis that SMase also enhances enterotoxin-mediated cytotoxicity in the human host (Oda et al., 2010; Doll et al., 2013).

Since *B. thuringiensis* has been reported to produce enterotoxins (Griffiths, 1990; Damgaard et al., 1996; Gaviria Rivera et al., 2000) and is routinely not differentiated from *B. cereus s.s.* by diagnostic laboratories, some outbreaks of food poisoning attributed to *B. cereus* may in fact have been caused by *B. thuringiensis*. This is of particular interest, since *B. thuringiensis* is widely used as biopesticide in organic farming on account of the pronounced insecticidal effects of crystal proteins (Cry toxins), which are formed during sporulation of *B. thuringiensis* (Bravo et al., 2011). Most biopesticide formulations used contain both insecticidal proteins and spores (EFSA BIOHAZ Panel, 2016).

Previous studies have demonstrated *B. thuringiensis* enterotoxin expression using immunological assays (Damgaard, 1995; Hansen and Hendriksen, 2001; Yang et al., 2003). However, these assays only recognize certain enterotoxin subunits (EFSA BIOHAZ Panel, 2016) and are not a suitable predictor of cytotoxicity (Miller et al., 2018). Data on *B. thuringiensis* enterotoxin production generated using bioassays are scarce. Damgaard et al. (1996) and Gaviria Rivera et al. (2000) were able to show that culture supernatants of *B. thuringiensis* isolates inhibited [^{14}C]-leucine uptake in a Vero cell assay. However, the authors only included three biopesticide strains HD-1 (serotype *kurstaki*), HD-567 (serotype *israelensis*), and NB-125 (serotype *tenebrionis*). In particular, ABTS-1857 (serotype *aizawai*), which has been discussed as causative agent of a foodborne outbreak in Germany in 2012 (EFSA BIOHAZ Panel, 2016), was missing.

In spite of these findings, the relevance of *B. thuringiensis* as causative agent of foodborne disease is controversially discussed (Jackson et al., 1995; McIntyre et al., 2008; EFSA BIOHAZ Panel, 2016; Raymond and Federici, 2017) and the EFSA recently published a scientific opinion (EFSA BIOHAZ Panel, 2016) stressing the urgent need for further data in order to improve risk assessment.

Therefore, in this study, we chose a systematic approach to gain further insights into the hazardous potential of naturally occurring and commercially used *B. thuringiensis*. To that end, a collection of *B. thuringiensis* isolates obtained from commercially used biopesticides, foods, and outbreaks was characterized using *panC* typing, *panC*-based SplitsTree analysis, toxin gene profiling, FTIR-spectroscopic analysis, a cytotoxicity assay, and a SMase assay.

MATERIALS AND METHODS

Bacterial Strains

This study includes a total of 39 *B. thuringiensis* isolates. Eight isolates from biopesticides (**Table 1**), 24 isolates from foods (**Table 2**), and seven isolates linked to three foodborne outbreaks (**Table 3**). *B. cereus sensu lato* were isolated from foods following standard routine procedures by plating samples either directly or after homogenization in serial dilutions on selective media (MYP) and with incubation at 30 and 37°C, respectively. For strains associated with outbreak investigations, additional information on the isolation context is provided elsewhere (EFSA BIOHAZ Panel, 2016; Schmid et al., 2016). Isolates from biopesticides were obtained by plating of serial dilutions of the respective biopesticides on MYP agar.

Screening for parasporal crystal bodies enabled *B. thuringiensis* species identification and was performed as follows: Isolates were grown on T3 agar (Travers et al., 1987) for 3 days at 30°C. Sporulated culture material was resuspended

TABLE 1 | Background information on the seven *Bacillus thuringiensis* biopesticide strains included in this study.

Biopesticide strain[a]	*B. thuringiensis* subspecies	Isolate ID in this study
GC-91	*aizawai*	CH_186
ABTS-1857	*aizawai*	CH_181
		CH_185
B401	*aizawai*	P05_2
SA-11	*kurstaki*	CH_164
ABTS-351	*kurstaki*	CH_183
Solbac	*israelensis*	CH_133
NB-176	*morrisoni* (var. *tenebrionis*)	CH_187

[a] *If no strain was specified on the product, trade names are given.*

TABLE 2 | Background information on the *Bacillus thuringiensis* isolates collected from food sources included in this study.

Strain ID	Food source	Isolation context
CH_9	Heated chicken breast	Army catering facility
CH_10	Heated tomatoes	Army catering facility
CH_19	Pork roast	Army catering facility
CH_24	Heated potatoes	Army catering facility
CH_26	Pollack filet & sauce	Army catering facility
CH_34	Runner beans	Army catering facility
CH_35	Ratatouille	Army catering facility
CH_40	Rosemary	Retail level
CH_41	Asia Mix (peppermint, coriander, thai chives)	Retail level
CH_42	Organic oregano	Retail level
CH_43	Organic sage	Retail level
CH_44	Organic peppermint	Retail level
CH_48	Rosemary	Retail level
CH_50	Organic coriander	Retail level
CH_65	Tarragon	Retail level
CH_66	Basil	Retail level
CH_69	Lasagna (precooked)	Surveillance
CH_72	Vegetable juice (spinach, carrot, cucumber, mint)	Surveillance
CH_81	Sauce (precooked)	Surveillance
CH_95	Sushi	Retail level
CH_96	Sushi	Retail level
CH_160	Heated pasta	Surveillance
P01_1	Honey	Self-surveillance
P01_3	Honey	Self-surveillance

TABLE 3 | Background information on the seven *Bacillus thuringiensis* isolates collected in association with outbreaks.

Outbreak	Strain ID	Sample	Source[a]	Year of isolation
Lower Austria	2/27/S	Human feces	Vetmeduni	2013
Lower Austria	6/27/S	Human feces	Vetmeduni	2013
Lower Austria	1/29 AGES	Fruit salad	AGES	2013
Linz	3/22 AGES	Bell pepper	AGES	2013
Germany	CVUAS 2492	Lettuce	CVUAS	2012
Germany	CVUAS 9660	Lettuce	CVUAS	2012
Germany	CVUAS 9659	Lettuce	CVUAS	2012

[a]*AGES, Austrian Agency for Health and Food Safety; CVUAS, Chemisches und Veterinäruntersuchungsamt Stuttgart; Vetmeduni, University of Veterinary MedicineVienna.*

in 10 μL sterile deionized water and immediately screened for parasporal crystals of diamond, bipyramidal, or spherical shape using phase contrast microscopy and oil immersion (EFSA BIOHAZ Panel, 2016).

panC Typing and *panC*-Based SplitsTree Analysis

All strains included in the study were subjected to *panC* typing for assignment to phylogenetic groups using the pantothenate synthetase gene as previously described (Guinebretière et al., 2008). Cluster analysis of *panC* nucleotide sequences was performed using the SplitsTree^{TM} software (http://www.splitstree.org). Several reference strains were included in the SplitsTree analysis (panC type I: DSM 12442; panC type II: WSBC10311; panC type III: Ames; panC type IV: ATCC 14579; panC type V: BCT-7112; panC type VI: WSBC 10204; panC type VII: NVH391-98).

FTIR Spectroscopic Fingerprinting

The strains were grown as lawns on tryptone soy agar (TSA) plates (Oxoid, Wesel, Germany) at 25°C for 24 h ± 30 min. Samples were prepared as described by previously (Oberreuter et al., 2002; Ehling-Schulz et al., 2005). Briefly, one loop of cell material was suspended in 100 μL sterile deionized water. Isolates yielding a clumpy suspension were subjected to ultrasonication for 5 × 1 s at 100% power with a Bandelin Sonopuls HD2200 (Bandelin electronic, Berlin) in order to improve spectral quality. Bacterial suspension were spotted on a zinc selenite (ZnSe) optical plate and dried at 40°C for 30 min. Infrared absorption spectra were recorded, using a HTS-XT microplate adapter coupled to a Tensor 27 FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany). Spectral acquisition was performed in transmission mode in the spectral range of 4,000–500 cm^{-1} using the following parameters: 6 cm^{-1} spectral resolution, zero-filling factor 4, Blackmann-Harris 3-term apodization, and 32 interferogramms were averaged with background subtraction for each spectrum. Independent measurements were prepared per strain to yield the number of spectra per strain required for cluster analysis. The quality of FTIR spectral data was evaluated first using OPUS software (version 7.5; Bruker Optics, Ettlingen, Germany). Additionally, second derivatives were calculated using the Savitzky-Golay algorithm with 11 smoothing points and the derivative spectra were unit vector normalized subsequently for further data processing. The spectral region of 1,500–800 cm^{-1} was chosen as fingerprint region and FTIR spectral data were subjected to hierarchical cluster analysis (HCA) using the Ward's algorithm. Further, the FTIR data set was tested against the normality of the data by using Mardia and Royston tests (Mecklin and Mundform, 2004). These resulted in a non-normality assumption (the degree of significance, $p < 0.0001$), which is considered as monotonic but also non-linear distribution. Thus, spearman rank correlation was utilized for analysis of this FTIR data set, showing non-parametric statistic monotonic association between the variables. Spectral pre-processing and multivariate data analysis of FTIR spectra were performed using the Unscrambler X (version 10.5, Camo AS, Norway) and Orange data mining toolbox for Python (software version 3.13.0; Demšar et al., 2013).

Toxin Gene Profiling

All isolates were screened for the presence of toxin genes *nheAB, hblDA, cytK,* and *ces*, coding for the non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl), cytotoxin K (CytK), and cereulide (Ces), respectively. PCR-based screening for toxin genes, as well

as subsequent assignment to toxin profiles A–G was performed as previously described (Ehling-Schulz et al., 2006).

The following strains were used as positive controls: F1942/85 for *nhe, hbl, cytK* (isolated from an outbreak of *B. cereus* diarrheal disease) and F4810/72 for *ces* (isolated from vomit in a clinical case of *B. cereus* emetic disease). Details of strains used as control are provided elsewhere (Ehling-Schulz et al., 2005).

Vero Cell Cytotoxicity Assay

Cytotoxicity in a Vero cell assay was used to determine enterotoxin production. An overnight culture of each isolate in 3 mL CGY (16–18 h, 30°C, 120 rpm) was used to adjust a 30 mL CGY day culture in an Erlenmeyer to an OD_{600} of 0.05. The day cultures were incubated at 30°C (120 rpm shaking) until an OD_{600} of 7 was reached. A volume of 5 mL of each day culture was centrifuged at 11,000 rpm for 10 min to harvest the supernatant. Sterile filtrated supernatant aliquots of 1 mL were supplemented with 10 μL 0.1 M EDTA-Na_2 and stored at −80°C. Cytotoxicity was subsequently determined using Vero cells as previously described (Moravek et al., 2006). The reciprocal titer of the reference strain NVH 0075-95, a *B. cereus* strain isolated from vegetable stew in a clinical case of *B. cereus* diarrheal disease in Norway in 1995 (Lund and Granum, 1996), was used for normalization of absolute values. Strains were classified as low level, mid level, or high level enterotoxin producers based on the classification described by Jeßberger et al. (2015), with cutoffs normalized based on the mean of the high level reference strain NVH 0075-95.

Sphingomyelinase Assay

Overnight cultures of all isolates (3 mL LB broth, 37°C, 120 rpm) were used to adjust 50 mL of LB broth to an OD_{600} of 0.05. All day cultures were subsequently grown to an OD_{600} of 4 at 37°C and 120 rpm shaking and harvested by centrifugation (6,500 × *g*, 4 min at 4°C). Cell pellets were discarded and supernatants were subjected to sterile filtration (pore size 0.2 μm). Six milliliters of sterile supernatant were concentrated to 100 μL using Vivaspin™ protein concentrator spin columns (GE Healthcare) with a cut-off size of 30,000 kDa. The sphingomyelinase (SMase) activity of the isolates was subsequently determined using the Amplex Red Sphingomyelinase Assay Kit (Invitrogen/Molecular Probes) in accordance with the manufacturer's instructions with minor changes: all reactions were developed for 20 min at 37°C in a light-protected 96-well microplate (Corning Costar Assay Plate, Sigma Aldrich). In total, only 0.1 μg of protein of each sample was used. Fluorescence was measured with extinction/emission wavelengths of 530/585 nm (SpectraMax® M3 Microplate Reader, Molecular Devices). Experiments were done in duplicate. To account for differences in protein concentrations between strains, the enzyme activity was normalized to the protein concentration of the respective supernatant using a Bradford-based protein assay (Roti-Quant, Carl Roth GmbH), resulting in enzyme activity expressed in mU per mg of protein. *B. cereus* strain NVH 0075-95 associated with a clinical case of *B. cereus* diarrheal disease (Lund and Granum, 1996) was used as a reference.

RESULTS

panC Typing and *panC* Based SplitsTree Analysis

All strains included in the study were subjected to *panC* typing for assignment to phylogenetic groups using the pantothenate synthetase gene as previously described (Guinebretière et al., 2008). A comprehensive overview of *panC* types detected is presented in **Table 4**. With the exception of strain CH_95 assigned to *panC* type V, all other *B. thuringiensis* isolates were assigned to *panC* type IV. SplitsTree analysis was used to allow for higher resolution based on *panC* nucleotide sequences and clustered the tested isolates in seven groups designated a-g (see **Figure 1**). Cluster a comprised biopesticides strains ABTS-1857, GC-91, and B401, as well as the isolates obtained in association with the outbreak potentially caused by consumption of salad in Germany in 2012 and two human feces isolates from diarrheal disease patients during an outbreak in Austria. In addition, the cluster comprised four food isolates. Cluster b comprised biopesticide strains SA-11 and ABTS-351, as well as 15 food isolates and two outbreak associated isolates from Austria originating from food. Cluster c comprised the strain isolated from biopesticide Solbac, cluster d comprised NB-176 and one food isolate, and clusters e, f, and g exclusively comprised one food isolate each.

FTIR Spectroscopic Analysis

Chemometric assisted FTIR spectroscopy was used to investigate the correlation between the FTIR spectra of *B. thuringiensis* bacterial strains isolated from different sources (biopesticides, food, and in association with foodborne outbreaks). This analysis revealed two main clusters, designated FTIR-A and FTIR-B (see **Figure 2**). Cluster FTIR-A comprised the *B. thuringiensis* ssp. *aizawai* strains. The biopesticide strain NB-176 (*B. thuringiensis* ssp. *morrisoni*) formed a singleton within this cluster. The two subclusters of the cluster FTIR-B can be assigned to *B. thuringiensis* ssp. *kurstaki* and *B. thuringiensis* ssp. *israelensis,* respectively. Although the biopesticide *B. thuringiensis* strains belonging to different serotypes clustered separately, they were intermixed with isolates from foods and foodborne outbreakes. For instance, the biopesticide *B. thuringiensis* ssp. *aizawai* strains were clearly separated from the serotypes *kurstaki* and *israelensis* but clustered together with isolates collected in the context of outbreak investigations (CVUAS2492, CVUAS9660, CVUAS9659, 2/27/S, 6/27/S) and isolates from foods (P01_1, P03_1) sprayed with *B. thuringiensis* ssp. *aizawai* directly before the harvest.

Toxin Gene Profiling

As revealed by toxin gene profiling (Ehling-Schulz et al., 2006), all *B. thuringiensis* isolates in this study harbored one or more enterotoxin genes (see **Table 4**). The *nhe* gene was detected in all isolates. All biopesticide and outbreak isolates were also positive for *hbl* and, with the exception of biopesticide strain NB-176, also for *cytK*. Isolates were assigned to toxin profiles A (91%; *nhe, hbl, cytK*), C (3%; *nhe, hbl*), D (3%; *nhe, cytK*), and F (3%; *nhe*). As expected for *B. thuringiensis,* all *cytK* positive isolates exhibited

TABLE 4 | Characterization results of the *Bacillus thuringiensis* isolates included in this study.

Source[a]	Isolate ID	Toxin profile[b,c]	SplitsTree cluster	FTIR cluster[d]	*panC typing*	Enterotoxin production in the Vero assay[e]			SMase production		
						Normalized reciprocal titer	Std. Dev.	Classification	Normalized amplex red result	Std. Dev.	Classification
P	CH_186	A	a	FTIR-A2	IV	0.3	0.0	Low	0.00	0.00	≤detection limit[f]
P	CH_181	A	a	FTIR-A1	IV	0.4	0.1	Mid	0.16	0.14	Low
P	CH_185	A	a	FTIR-A1	IV	0.5	0.2	Mid	0.06	0.05	Low
P	P05_2	A	a	FTIR-A1	IV	0.4	0.0	Mid	0.13	0.03	≤detection limit[f]
P	CH_164	A	b	FTIR-B2	IV	0.8	0.1	Mid	0.00	0.00	≤detection limit[f]
P	CH_183	A	b	FTIR-B2	IV	0.4	0.1	Mid	0.00	0.00	≤detection limit[f]
P	CH_133	A	c	FTIR-B1	IV	0.8	0.3	Mid	0.04	0.03	≤detection limit[f]
P	CH_187	C	d	FTIR-S	IV	0.2	0.0	Low	0.00	0.00	≤detection limit[f]
F	CH_9	D	b	FTIR-B2	IV	0.4	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_10	D	b	FTIR-B2	IV	0.4	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_19	D	b	FTIR-B2	IV	0.3	0.0	Low	0.00	0.00	≤detection limit[f]
F	CH_24	D	b	FTIR-B2	IV	0.4	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_26	F	b	FTIR-A1	IV	0.5	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_34	A	b	FTIR-B2	IV	0.2	0.0	Low	0.00	0.00	≤detection limit[f]
F	CH_35	A	b	FTIR-A1	IV	0.1	0.0	Low	0.00	0.00	≤detection limit[f]
F	CH_40	A	b	FTIR-B2	IV	0.8	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_41	A	b	FTIR-A1	IV	0.8	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_42	A	b	FTIR-B2	IV	0.7	0.0	Mid	0.00	0.00	≤detection limit[f]
F	CH_43	A	b	FTIR-A1	IV	0.8	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_44	A	b	FTIR-B2	IV	0.6	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_48	C	d	FTIR-B2	IV	1.5	0.3	High	1.21	0.12	High
F	CH_50	A	g	FTIR-A2	IV	0.5	0.1	Mid	0.60	0.42	Medium
F	CH_65	A	a	FTIR-A2	IV	0.2	0.0	Low	0.00	0.00	≤detection limit[f]
F	CH_66	A	a	FTIR-A2	IV	0.4	0.0	Mid	0.00	0.00	≤detection limit[f]
F	CH_69	A	b	FTIR-B1	IV	0.5	0.1	Mid	0.00	0.00	≤detection limit[f]
F	CH_72	A	a	FTIR-A1	IV	0.5	0.1	Mid	0.14	0.11	Low
F	CH_81	A	e	FTIR-A1	IV	0.7	0.1	Mid	0.01	0.02	Low
F	CH_95	F	f	FTIR-A2	V	0.2	0.0	Low	0.08	0.08	Low
F	CH_96	A	b	FTIR-B2	IV	0.4	0.0	Mid	1.14	0.16	High
F	CH_160	A	b	FTIR-A1	IV	0.7	0.0	Mid	0.00	0.00	≤detection limit[f]
F	P01_1	A	a	FTIR-A1	IV	0.7	0.0	Mid	0.11	0.01	Low
F	P03_1	A	a	FTIR-A1	IV	0.6	0.1	Mid	0.14	0.01	Low
O	2/27/S	A	a	FTIR-A1	IV	0.5	0.1	Mid	0.15	0.10	Low
O	6/27/S	A	a	FTIR-A1	IV	0.5	0.2	Mid	0.14	0.11	Low
O	1/29 AGES	A	b	FTIR-A1	IV	0.3	0.1	Low	0.00	0.00	≤detection limit[f]
O	3/22 AGES	A	b	FTIR-B2	IV	0.3	0.1	Low	0.00	0.00	≤detection limit[f]
O	CVUAS 2492	A	a	FTIR-A1	IV	0.4	0.0	Mid	0.12	0.09	Low
O	CVUAS 9660	A	a	FTIR-A1	IV	0.4	0.0	Mid	0.16	0.14	Low
O	CVUAS 9659	A	a	FTIR-A1	IV	0.4	0.0	Mid	0.30	0.25	Low

[a] *Isolates obtained from pesticides (P), foods (F), or in association with outbreaks (O).*

[b] *Toxin profiles A–G correspond to the presence of the following combinations of toxin genes: A, nhe, hbl, cytK; B, nhe, cytK, ces; C, nhe, hbl; D, nhe, cytK; E, nhe, ces; F, nhe; G, cytK.*

[c] *All cytK positive isolates detected in this study harbored cytK-2.*

[d] *Singleton (S) in the cluster A.*

[e] *Values represent absolute values normalized using the absolute value of the highly toxic reference strain NVH 0075/95 included in the same run. Strains were classified as low level, mid level, or high level enterotoxin producers in adaptation of Jeßberger et al. (2015): low < 0.4, mid = 0.4–0.8, high > 0.8.*

[f] *The limit of detection was determined using a SMase dilution series. The lowest amount of SMase that yielded a positive test result after 20 min was 0.444 mU, with one unit of SMase being defined as the amount of SMase that will hydrolyse 1 μmol of TNPAL-sphingomyelin per minute (at pH 7.4 and 37°C). SMase levels of ≤0.400 mU yielded a negative result under the same test conditions.*

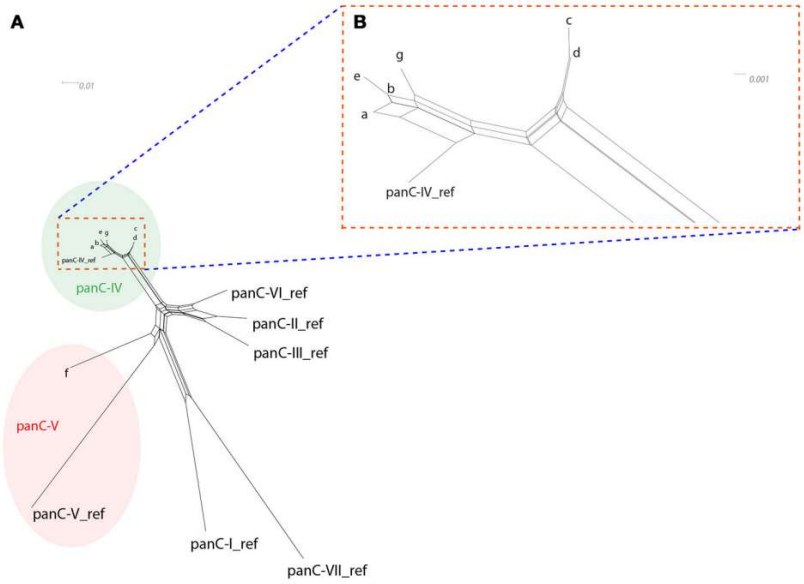

FIGURE 1 | SplitsTree depicting the relatedness of the tested isolates based on *panC* sequence variations (**A**: overview over full SplitsTree, **(B)** detail zooming in on region depicting isolates assigned to *panC* type IV). SplitsTree generates unrooted phylogenetic networks from molecular sequence data. Proximity is used as an indicator of sequence similarity and thus relatedness of the respective isolates. Each of the 37 *B. thuringiensis* isolates exhibited one of only seven different *panC* nucleotide sequences. To improve readability of the network, these seven sequence variations are presented as clusters a-g. With the exception of one strain assigned to *panC* type V (CH_95, cluster f), all strains were assigned to *panC* type IV and clustered closely together, indicating that these isolates are closely related.

the *cytK-2* variant of the cytotoxin K gene. The *ces* gene encoding the emetic toxin cereulide was not detected in any of the isolates.

Cytotoxicity

All *B. thuringiensis* isolates included in this study showed cytotoxic effects in a Vero cell assay routinely used to assess the enterotoxicity of *B. cereus*. Although most isolates were classified as low or mid level enterotoxin producers, one isolate from rosemary (CH_48) exhibited cytotoxic effects 1.5x higher than the reference strain NVH 0075-95, known for its high-level enterotoxin production. An overview of all reciprocal titers determined in the Vero cell cytotoxicity assay is provided in **Figure 3**.

SMase Assay

An enzymatic assay was used to test all *B. thuringiensis* strains included in this study for their SMase activity. The reference strain NVH 0075-95, which is not only known for its high level of enterotoxin production but also for its high SMase activity (Doll et al., 2013), was included as a reference. All tested isolates, except three isolates from food (CH_48, CH_50, and CH_96), exhibited either no detectable SMase activity (detection limit: 0.004 U/mL) or produced low levels of SMase (**Figure 4**). *B. thuringiensis* ssp. *aizawai* strains ABTS-1857 and B401 as well as the *B. thuringiensis* ssp. *israelensis* isolate from Solbac showed low levels of SMase activity, while the other biopesticide strains tested negative.

DISCUSSION

Toxin gene profiling of the *B. thuringiensis* isolates obtained from biopesticides, foods, and outbreaks tested in this study revealed that the isolates commonly harbored enterotoxin genes. This is consistent with previous studies reporting high prevalences of enterotoxin genes in *B. thuringiensis* originating from pasteurized milk (Zhou et al., 2008), rice (Ankolekar et al., 2009; Kim et al., 2014), organic vegetables (Kim et al., 2017), food (Rosenquist et al., 2005; Ngamwongsatit et al., 2008), and soil (Ngamwongsatit et al., 2008). The collected evidence suggests that enterotoxin genes are common among *B. thuringiensis* independent of their source, and including biopesticide and food strains.

Several studies have confirmed that the *B. thuringiensis* strains from various sources express Nhe and/or Hbl using commercially available enterotoxin immunoassays (Abdel-Hameed and Landén, 1994; Damgaard, 1995; Hansen and Hendriksen, 2001; Rosenquist et al., 2005; Ankolekar et al., 2009; Kim et al., 2014). Damgaard (1995) screened various *B. thuringiensis* based biopesticides Bactimos, DiPel, Florbac FC, Foray 48B, MVP, Novodor FC, Turex, VecTobac, and XenTari for the presence of diarrheal enterotoxins using the *Bacillus* diarrhoeal enterotoxin visual immunoassay (BDE-VIA) kit provided by Tecra (Tecra diagnostics, Roseville, Australia). With the exception of one biopesticide, which lacks viable *B. thuringiensis* spores, all biopesticidal products yielded a positive result (Damgaard, 1995). Hansen and Hendriksen used

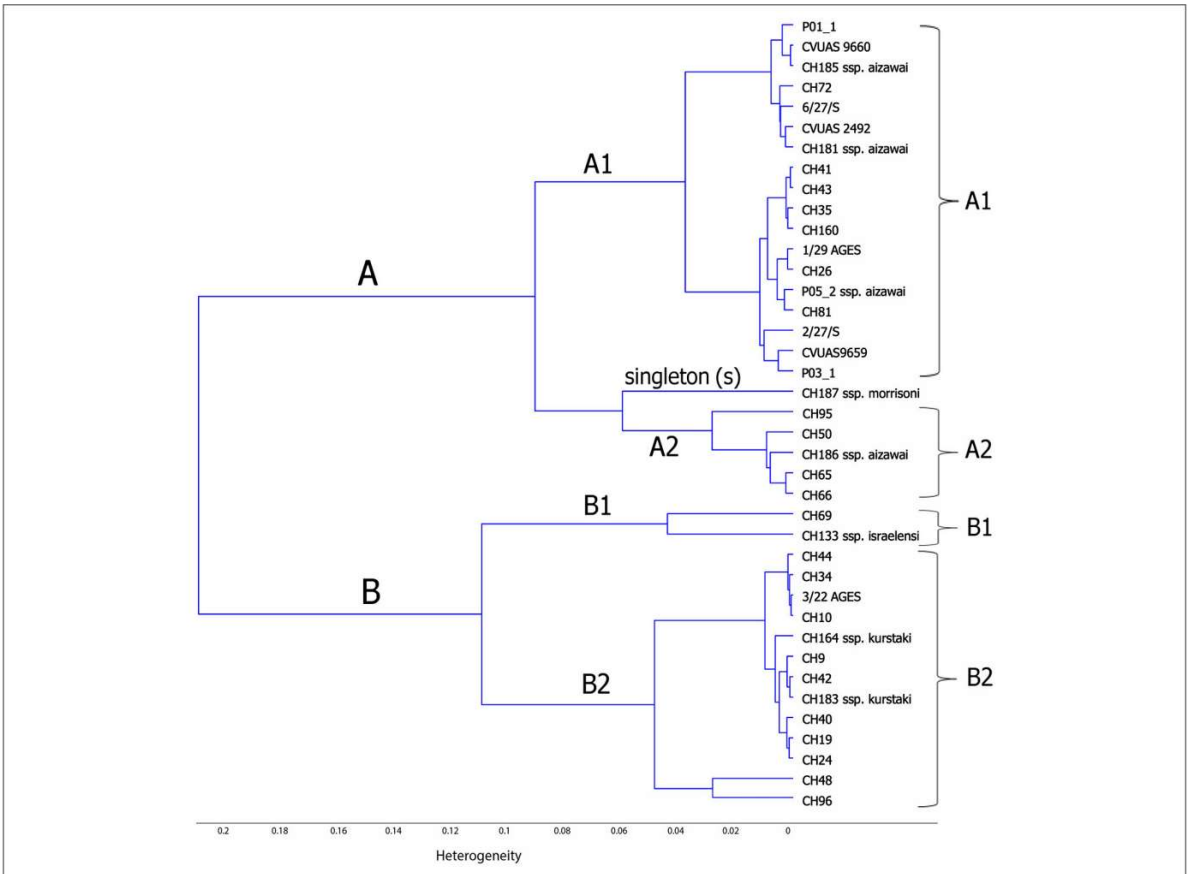

FIGURE 2 | Dendrogram depicting similarity of FTIR spectra of *B. thuringiensis* isolated from different biopesticides, foods, and in connection to foodborne outbreaks. This analysis revealed two main clusters, designated FTIR-A and FTIR-B. Cluster FTIR-A comprised the *B. thuringiensis* ssp. aizawai strains. The biopesticide strain NB-176 (*B. thuringiensis* ssp. morrisoni) forms a singleton within this cluster. The two subclusters of FTIR-B can be assigned to *B. thuringiensis* ssp. *kurstaki* and *B. thuringiensis* ssp. israelensis, respectively.

the same assay to demonstrate Nhe expression in biopesticide strains HD-1 (Dipel ES) and HD-567 (Hansen and Hendriksen, 2001). Rosenquist et al. (2005) showed that strains contained in the biopesticides Dipel, Bactimos, and Vectobac all tested positive for both Nhe and Hbl expression using BDE-VIA (Tecra diagnostics) and the *B. cereus* enterotoxin reverse passive latex agglutination kit (BCET-RPLA, Oxoid, Basingstoke, UK), respectively. However, it was not further specified if Dipel ES (*B. thuringiensis* ssp. *israelensis*, strain HD-1) or Dipel DF (*B. thuringiensis* ssp. *kurstaki*, strain ABTS-351) was tested.

Cell culture assays have been reported to allow for more sensitive detection of *Bacillus* diarrheal enterotoxins than immunological assays (Buchanan and Schultz, 1994) and to enable classification of strains into low, mid, and high level enterotoxin producers (Jeßberger et al., 2015). Information on *B. thuringiensis* enterotoxicity based on cell culture assays is scarce and the few studies published so far (Damgaard et al., 1996; Gaviria Rivera et al., 2000) comprised only few biopesticide strains. Damgaard et al. (1996) tested food isolates from pasta ($n = 5$), pitta bread ($n = 1$) and milk ($n = 1$), as well as the three biopesticide strains HD-1, NB-125, and HD-567 representing *B. thuringiensis* serotypes *kurstaki, tenebrionis,* and *israelensis,* respectively. It could be demonstrated that all culture supernatants, except that from one strain isolated from milk, inhibited protein synthesis in a Vero cell assay. Our current study adds Vero cytotoxicity data for various other biopesticide strains including ABTS-1857 (serotype *aizawai*), which has been discussed as causative agent of a foodborne outbreak in Germany in 2012 (EFSA BIOHAZ Panel, 2016).

In recent years, it has become increasingly evident that specific host settings and parameters are playing a crucial role for enteropathogenicity of *B. cereus*. In particular, the role of spore

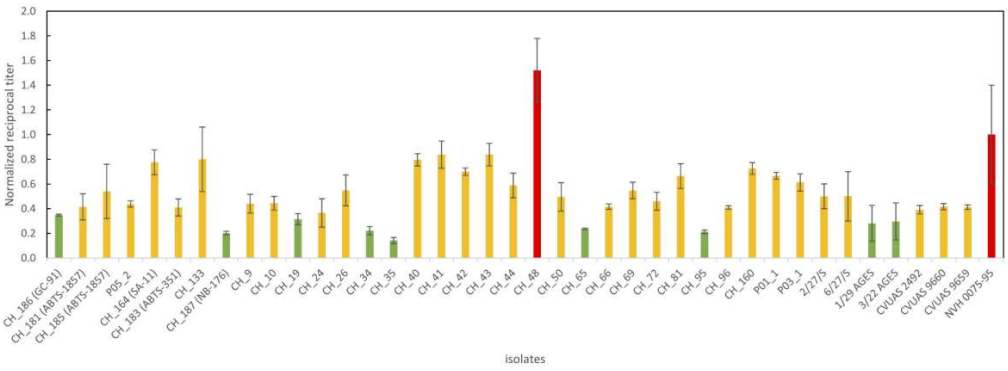

FIGURE 3 | Overview of Vero cell cytotoxicity used to determine enterotoxin production. Values represent absolute values normalized using the absolute value of the highly toxic reference strain NVH 0075/95 included in the same run. The majority of the *B. thuringiensis* isolates including most biopesticide strains and the isolates linked to the outbreak in Germany in 2012 were classified as medium level enterotoxin producers (indicated in yellow). One food isolate was classified as high level enterotoxin producer surpassing the cytotoxicity of the highly toxic reference strain by a factor of 1.5 (indicated in red). Several isolates, including the biopesticide strains GC-91 and NB-176 were classified as low level enterotoxin producers (indicated in green).

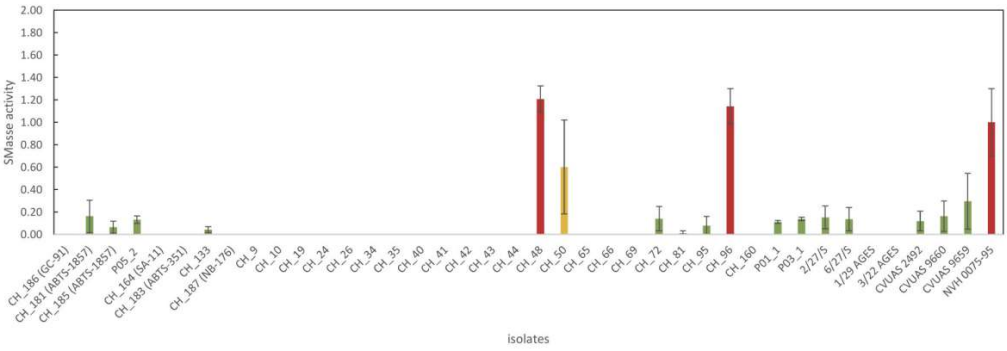

FIGURE 4 | Overview of sphingomyelinase production of the *B. thuringiensis* isolates. Values represent Amplex Red results normalized using NVH 0075/95 (high-level SMase producer). For the majority of *B. thuringiensis* isolates, no SMase production was detectable (indicating that ≤0.400 mU of SMase were produced). Two isolates from food (CH_48 from rosemary and CH_96 from sushi) produced high levels of SMase. Interestingly, CH_48 also produced massive amounts of enterotoxins, surpassing the cytotoxicity of the highly toxic reference strain by a factor of 1.5.

survival, germination, and adhesion under conditions mimicking the host and the impact of intestinal conditions on enterotoxin synthesis have been investigated (Wijnands et al., 2009; Berthold-Pluta et al., 2015; Jeßberger et al., 2017). However, further studies will be needed to fully understand the relationship of enterotoxin formation, *in vitro* cytotoxicity, and the ability of a strain to cause clinical disease.

With one exception, all *B. thuringiensis* isolates tested in this study were assigned to *panC* type IV, including all biopesticide strains. As *B. thuringiensis* strains have previously been reported in association with *panC* types II, III, IV, V, and VI (Guinebretière et al., 2008, 2010; Carroll et al., 2017), the close genetic relatedness observed among the biopesticide strains and isolates collected from foods in our study foster the hypothesis that biopesticide strains can indeed be detected on foodstuff. Comparative genomics of the *B. thuringiensis* population showed that strains that belong to the so-called clade 2, which comprises strains of *panC* types IV and V (Ehling-Schulz and Messelhäusser, 2014), possesss highly potent insecticidal toxins and carry multiple *cry* genes (Zheng et al., 2017; Méric et al., 2018). Due to their high invertebrate toxicity, these *B. thuringiensis* strains are ideal candidates for biopesticides and strains commonly used as biopesticides belong to this phylogenomic group. Consistent with this hypothesis, *panC* IV strains frequently originate from natural environments (soil, water, air, plants), various foods, and from insects (Guinebretière et al., 2008). Nevertheless, *panC* IV strains can also be found associated with foodborne outbreaks of diarrheal disease (Guinebretière et al., 2008; Jeßberger et al., 2015; Glasset et al., 2016). Food poisoning risk however, has been suggested to be highest for *B. cereus* group isolates that

belong to *panC* group III (Guinebretière et al., 2010), a group not detected among any of the isolates tested in this study. Growth temperature ranges vary between *panC* groups, with group IV being classified as mesophilic (10–45°C) and group V being classified as moderately psychrotolerant (8–40°C) (Guinebretière et al., 2008). *B. cereus* group strains assigned to *panC* IV have been shown to particularly frequently harbor *cytK* (Miller et al., 2018). Consistent with the findings in this study, Caco2 cytotoxicity of *panC* IV strains has been reported to vary greatly (Guinebretière et al., 2008; Jeßberger et al., 2015; Miller et al., 2018).

The relatedness of the isolates characterized in this study was further assessed using *panC*-based SplitsTree and FTIR spectroscopic analysis. The clusters obtained using these techniques, as well as the combined information derived from toxin gene profiling, *panC* typing, and cytotoxicity and SMase assays were used to determine characterization patterns. Several biopesticide strains exhibited characterization patterns that could not be distinguished from those originating from food or outbreak isolates (**Table 5**). The patterns obtained from *B. thuringiensis* isolated in the German outbreak in 2012 associated with lettuce previously treated with ABTS-1857 (XenTari) were identical to the one obtained from ABTS-1857. This is consistent with FTIR data generated by the authorities (EFSA BIOHAZ Panel, 2016). In addition, the *B. thuringiensis* from B401 used to control bee pests was indistinguishable from isolates obtained from self-surveillance food samples of a honey producer, and GC-91 (Agree) was indistinguishable from one food isolate. B. thuringiensis isolates were detected at a level of 3 × 104 cfu/g in the salad sample implicated in the outbreak in Germany in 2012 (EFSA BIOHAZ Panel, 2016) and at the same levels of 3 × 104 CfU/g in the honey samples from self surveillance (this study), emphasizing that *B. thuringiensis* used as biopesticide can enter the food production and be found in foods at retail level at high levels. In both cases the biopesticides were applicated directly before harvest.

B. thuringiensis based biopesticides were developed as a non-toxic alternative to chemical pesticides. They have been successfully applied in large-scale pest eradication for decades and allowed for a significant reduction of the use of chemical insecticides (Bravo et al., 2011). There are indications that enterotoxins are expressed by *B. thuringiensis* during septicemia in a target insect and therefore may contribute to the insecticidal effect of pesticides that contain not only crystal proteins, but also the organism itself (Kyei-Poki et al., 2007). Biopesticides exclusively relying on the insecticidal effects of purified *B. thuringiensis* crystals represent a safe alternative to formulations containing both crystals and viable endospores and have no known adverse effects on human health.

Our findings show that many *B. thuringiensis* biopesticide strains exhibit mid-level cytotoxicity in a Vero cell assay and that some of these strains cannot be differentiated from isolates obtained from foods or associated with outbreaks. Thus, we demonstrate that the use of *B. thuringiensis* strains as biopesticides may represent a food safety risk, underlining the importance of assessing the hazardous potential of each strain and formulation used. However, our findings also provide a novel explanation for the low number of clinical cases of diarrheal disease linked to *B. thuringiensis* over the last decades. With the exception of the low level enterotoxin producers GC-91 and NB-176, all other *B. thuringiensis* biopesticide strains tested exhibited mid level enterotoxicity. Nevertheless, compared to *B. cereus s.s.* their hazardous potential may be limited due to the lack of SMase, an important virulence factor complementing Nhe and Hbl induced cytotoxicity. We did not detect SMase production in the biopesticide strains GC-91, SA-11, ABTS-351, and NB-176. By contrast, biopesticide strains B401, ABTS-1857, and Solbac produced low levels of SMase. This is particularly interesting, as ABTS-1857 was implicated in the salad-related outbreak in Germany in 2012. Thus, further research should be focused on fully understanding the role of SMase in enteropathogenicity of *B. cereus s.l.*. Such research will not only lead to a better

TABLE 5 | Overview of biopesticide isolates and identical characterization patterns (toxin gene profile, *panC* type, *panC*-based SplitsTree cluster, FTIR cluster, cytotoxicity in a Vero cell assay, and SMase activity) determined for food or outbreak isolates.

Biopesticide strain[a]	Toxin profile[b]	*panC* type	SplitsTree cluster	FTIR cluster	Enterotoxin production in Vero assay	SMase production	*n* food isolates with identical pattern	*n* outbreak isolates with identical pattern
GC-91	A	IV	a	FTIR-A2	Mid	≤detection limit	1 (tarragon)	0
ABTS-1857 and B401[c]	A	IV	a	FTIR-A1	Mid	Low	3 (vegetable juice, 2 honey samples)	5 (3 salad samples Germany 2012; 2 human feces samples Austria 2013)
SA-11 and ABTS-351[d]	A	IV	b	FTIR-B2	Mid	≤detection limit	3 (spices)	0
Solbac	A	IV	c	FTIR-B1	Mid	≤detection limit	0	0
NB-176	C	IV	d	FTIR-S	Low	≤detection limit	0	0

Nineteen isolates exhibited a pattern not identified in a biopesticide strain and were therefore not included in this table.

[a] *In case no strain ID was provided on the product, trade names are used.*

[b] *Toxin profiles A–G correspond to the presence of the following combinations of toxin genes: A, nhe, hbl, cytK; B, nhe, cytK, ces; C, nhe, hbl; D, nhe, cytK; E, nhe, ces; F, nhe; G, cytK.*

[c] *Both ABTS-1857 and the B401 biopesticide isolates included in this study exhibited the same characterization pattern, which was detected again in isolates from foods and outbreaks.*

[d] *SA-11, ABTS-351, and three isolates from spices exhibited identical characterization patterns.*

understanding of the mechanisms of enteropathogenicity in *B. cereus*, but could also contribute to a better risk assessment of *B. thuringiensis* strains used as biopesticides.

CONCLUSION

We demonstrated that most *B. thuringiensis*—including most biopesticide strains—tested in this study represent mid level enterotoxin producers. Several biopesticide strains could not be differentiated from isolates obtained from foods or associated with outbreaks based on *panC* type, SplitsTree and FTIR analysis, toxin gene profiles, cytotoxicity, and SMase production. Our data therefore suggests that biopesticide strains may be detected on foods after harvesting and that *B. thuringiensis* based biopesticides may pose a risk to consumer health. However, we also hypothesize that the hazardous potential of many commercially used *B. thuringiensis* strains might be limited due to low SMase production. The data presented in this study are a crucial contribution toward improved risk assessment of foodborne *B. thuringiensis*.

AUTHOR CONTRIBUTIONS

SJ and ME-S conceived and designed the study. SJ, EK, NH, and RS carried out the experiments. PB, SG, RS, and MC contributed strains. MB performed the chemometric analysis of FTIR spectral data, SJ and ME-S analyzed and interpreted the data. SJ and ME-S wrote the manuscript. All authors revised and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4. Discussion

The work carried out in this PhD project revealed new insights into the regulation of pathogenicity of emetic *B. cereus*. The main part of the PhD thesis was to elucidate the role of the transcriptional regulator PagR in emetic *B. cereus* highlighting the protein's significant influence of cereulide toxin synthesis. The second part of this PhD thesis was the use of Äkta™ pure coupled with RPC as a new diagnostic tool for cereulide detection, purification and quantification, and the third part of this PhD thesis revealed the enteropathogenic properties of a variety of *B. thuringiensis* strains with a harmful potential to consumer health.

4.1 The transcriptional regulator PagR and its role in the regulatory network of emetic *B. cereus*

The relationship of *B. anthracis* to a parental *B. cereus* subgroup is reflected by the evolution of the conserved state of the pXO1-like plasmids and plasmid pXO1 (Keim et al., 1997; Helgason et al., 2000; Baillie and Read, 2001; Pannucci et al., 2002). The genetic organization of the virulence genes of plasmid pXO1 is similar to the pathogenicity islands located on the chromosome of other bacterial pathogens (Okinaka et al., 1999). In previous studies, it was stated that *B. anthracis* can be classified as a part of the *B. cereus* group due to the potential of horizontal gene transfer within the group itself and their virulence (Helgason et al., 2000). Moreover, it was demonstrated that *B. thuringiensis* and *B. cereus* can exchange their plasmids in different food sources (Van der Auwera et al., 2007). A direct exchange among the members of the *B. cereus* group was demonstrated by the occurrence of pXO1 homologous fragments in *B. anthracis, B. thuringiensis* and *B. cereus* (Hu et al., 2006).

In *B. anthracis*, pathogenicity islands of 44.8 kb are surrounded by IS1617 elements in inverted orientation containing the *lef, cya, pagA, atxA,* and *pagR* genes (Okinaka et al., 1999; Tucker and Ballard, 2005). The cereulide toxin gene cluster of emetic *B. cereus*, a ~46 kb region, is the most distinguishing feature of megaplasmid pCER270 (Rasko et al., 2007). The *ces* gene cluster of emetic *B. cereus* is flanked upstream and downstream with similar regions of 85 % to 95 % nucleotide conservation to most of the pXO1-like plasmids with a following 18 kb region uniquely present in the megaplasmid pCER270 (Rasko et al., 2007).

In silico research for putative plasmid-encoded transcription factors revealed two novel transcriptional regulators designated PagR and PagR1 of emetic *B. cereus* (also named PagRBc and PagR1Bc), which are homologues to PagR of *B. anthracis* (Kalbhenn et al., 2022). The amino acid sequence of PagR of emetic *B. cereus* was compared to PagR of *B. anthracis* and resulted in a high identity of 63.6 % and similarity of 78.8 % (Kalbhenn et al., 2022). Whereas, PagR1 of emetic *B. cereus* reveals 95.9 % identity and 99.0 % similarity to PagR1 of *B. anthracis* on the pXO1 plasmid (Kalbhenn et al., 2022). The *ces* operon is located on the megaplasmid pCER270 with a distance of 22 kb to *pagR1Bc* and 102.5 kb to *pagRBc* (Kalbhenn et al., 2022). Previous studies showed that further homologous genes were expected (Rasko et al., 2007), thus hypothesizing that other PagR-like transcriptional regulators will be found on plasmids and chromosomes in both microorganisms *B. anthracis* and *B. cereus*. Based on this high sequence homology between the PagR homologues, it can be assumed that the plasmid-encoded transcriptional regulators PagR of emetic *B. cereus* and *B. anthracis* have the same functions and regulate the toxin synthesis (Kalbhenn et al., 2022).

A Basic Local Alignment Search Tool (BLAST) of the sequence of the plasmid-encoded transcriptional regulator PagR revealed additional PagR homologues in *B. thuringiensis, B. mycoides, B. wiedmannii*, *B. toyonensis*, *B. paranthracis*, and other members of the *B. cereus sensu lato* group indicating that these members of the ArsR/SmtB family are indeed homologues. Moreover, similar PagR proteins with a helix-turn-helix motif (HTH) exist not only in the *B. cereus sensu lato* group but also in other microorganisms such as *Salmonella Typhimurium,* in which the master regulator PagR is involved in a complex regulatory mechanism of *Salmonella* pathogenicity island 2 gene expression at low phosphate and magnesium levels (Jiang et al., 2020). Another example is PagR of *Pantoea agglomerans*, which has characteristic autoinducer bindings and affects a quorum-sensing system (Chalupowicz et al., 2008).

Based on the significant similarities of *B. anthracis* plasmid pXO1 to emetic *B. cereus* pXO1-like plasmid, this main part of the PhD thesis focused on dissecting the role of the novel PagR-like transcriptional regulator, termed PagR (PagRBc), in the regulatory network of emetic *B. cereus* (Figure 4), (Kalbhenn et al., 2022). The role of the other PagR-like transcriptional regulator, designated PagR1 of emetic *B. cereus,* need to be dissected in future studies.

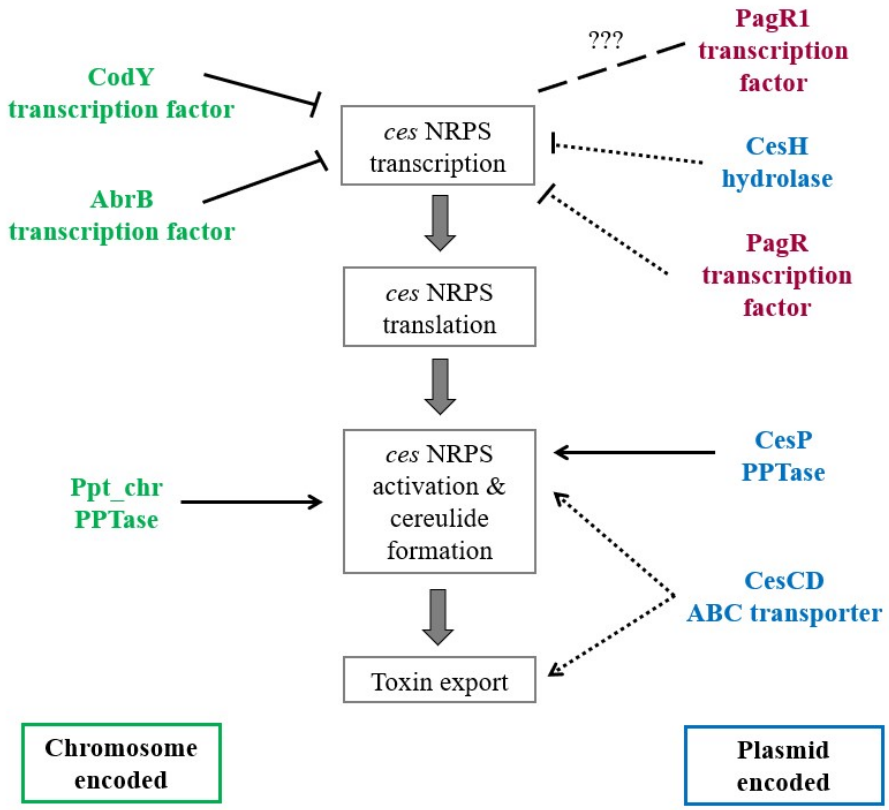

Figure 4: Schematic representation of the regulation of cereulide toxin synthesis in emetic *B. cereus*. The interplay of chromosome and plasmid-encoded factors control the cereulide toxin synthesis in a multilayered process at several levels (Kalbhenn et al., 2022). The novel transcriptional regulator PagR is a member of the ArsR/SmtB family and exerts the control of *ces* transcription as a repressor. The function and its influence on cereulide toxin synthesis of the other homologous transcriptional regulator PagR1 remains unexplored. Figure modified according to Lücking et al., 2015.

The transcriptional master regulator CodY of emetic *B. cereus* is described as the regulator interlinking toxin synthesis and enterotoxin synthesis with the general metabolism (Frenzel et al., 2012). CodY of *B. anthracis* is a direct repressor of the S-layer genes *sap* and *eag* and has a significant influence on the central metabolism as well as is associated with other transcriptional regulators dependent on specific environmental conditions (Chateau et al., 2013; Kim et al., 2016). The putative hydrolase CesH of emetic *B. cereus* is upregulated in the later growth phases during *ces* expression (Ehling-Schulz et al., 2006a; Lücking et al., 2015).

Recently, CesH was described as an α/β-fold hydrolase that could be translated into an esterase capable of neutralizing the negative effects of cereulide on the ionophore-producing host itself (Tian et al., 2019). The chromosome-plasmid crosstalk has a high importance in *B. anthracis* and *B. cereus* influencing toxin synthesis and virulence (Perego and Hoch, 2008; Lücking et al., 2015). Previous studies have described similar cross-regulations in other bacterial species too (Riffaud et al., 2020).

The plasmid-encoded transcriptional regulator PagR of *B. anthracis* negatively controls *pagA* and binds to the *pagAR* promoter region (Mignot et al., 2003). Furthermore, *pagR* protects the promoter regions of the chromosome-encoded S-layer gene *sap* in a symmetrically manner and of the chromosome-encoded S-layer gene *eag* in an asymmetrically manner, and is thus involved in their regulation (Mignot et al., 2003; Corsi and Koehler, 2022). Based on this, further studies should investigate the role of the transcriptional regulator PagR regulating the S-layer genes *sap* and *eag* of emetic *B. cereus* to gain a deeper knowledge of the regulatory mechanism with the aim to avoid foodborne outbreaks. (Kalbhenn et al., 2022). Supporting this notion, the results of the EMSA showed that the plasmid-encoded PagR of emetic *B. cereus* acts as a transcriptional repressor and binds *in vitro* directly to the *ces* promoter region in comparison to the same binding affinity of PagR of *B. anthracis* to the *ces* promoter region (Kalbhenn et al., 2022). A comparable binding affinity of both PagR homologous was observed for the *eag* promoter region of emetic *B. cereus* and *B. anthracis* (Kalbhenn et al., 2022). Not only the structural similarities of PagR of emetic *B. cereus* and *B. anthracis* but also the similar binding affinities to the *ces* and *eag* promoter regions imply that both *pagR* genes are homologous in their function (Kalbhenn et al., 2022). In addition, tetrameric protein-DNA complexes are formed by PagR of emetic *B. cereus* and its homologous to protect the DNA from transcription processes *in vivo* (Kalbhenn et al., 2022).

The protein structure of PagR in emetic *B. cereus* has all characteristic features of the ArsR/SmtB family members such as the winged HTH-DNA-binding domain, the two β-sheets forming the hairpin structure and the homodimer formation (Zhao et al., 2010; Kalbhenn et al., 2022). Fostering the hypothesis, that both PagR transcriptional regulators are structurally similar and share the same functionality (Kalbhenn et al., 2022). Results of the BACTH experiments showed that the transcriptional regulator PagR of emetic *B. cereus* has stronger

interactions *in vivo* with PagR of *B. anthracis* reflecting the structural homologies (Kalbhenn et al., 2022). The transcriptional regulator PagR tightly controls the *ces* expression leading to an enhanced cereulide synthesis in the early growth phases with a constant level of high *ces* transcripts during the different growth phases (Kalbhenn et al., 2022). According to the recent results, the transcriptional repressor PagR can form stable dimers mediating strong-protein DNA interactions (Kalbhenn et al., 2022). Furthermore, it was shown that the homologue PagR of *B. anthracis* can substitute the function of the indigenous PagR of emetic *B. cereus* in cereulide synthesis (Kalbhenn et al., 2022). The *pagR* gene of *B. anthracis* is transcribed bicistronically, whereas the *pagR* homologue of the emetic *B. cereus* is transcribed monocistronically (Hoffmaster and Koehler, 1999a; Corsi and Koehler, 2022; Kalbhenn et al., 2022).

Recently, a novel PagR-like paralog designated PagR2 located on the plasmid pXO2 of *B. anthracis* was found with 71 % identity of amino acid sequence to PagR of the *pagAR* operon on plasmid pXO1 (Corsi and Koehler, 2022). A new concept of the adaptation of specific isolates to different niches states that specific phenotypic differences may be caused by altered gene expression of a variety of trans-acting factors rather than loss or increase of specific coding regions (Toby et al., 2014; Okinaka and Keim, 2016). Therefore, it is mandatory to decipher the multilayered process of different other “trans-acting” PagR homologues and paralogs in *B. anthracis* as well as emetic *B. cereus* to gain a better understanding of toxin synthesis, virulence mechanisms and pathophysiology.

The *pagR2* gene located on the pXO2 plasmid of *B. anthracis* showed its involvement in the virulence mechanisms and contribution to lethal levels of toxin production of *B. anthracis* emphasizing a plasmid-plasmid crosstalk (Liang et al., 2016). Recently published results showed that *pagR* of plasmid pXO1 of *B. anthracis* is involved in the capsule synthesis, whereas the *cap* genes are located on the plasmid pXO2 (Liang et al., 2016, 2017). A general similarity of chromosomal genes of *B. cereus* and *B. anthracis* contributing to pathogenicity comprising phospholipases, iron acquisition systems and hemolysins were determined (Rasko et al., 2007; Read et al., 2003).

The master regulator AtxA is responsible for the virulence, toxin gene expression, and control of sporulation in *B. anthracis* (Mignot et al., 2004; Dale et al., 2018; McCall et al., 2019). The transcriptional regulator PagR conveys the effect of AtxA on *sap* and *eag,* and AtxA binds directly to the promoter region of *pagA* (Mignot et al., 2004; McCall et al., 2019). PagR of *B. anthracis* is described as an intermediate effector of the AtxA signalling cascade in gene expression (Mignot et al., 2004). A *pagR* deletion mutant of *B. anthracis* showed an increase of the *atxA* expression level leading to the hypothesis that PagR might bind to the promoter region of *atxA* (Hoffmaster and Koehler, 1999a; Zhao et al., 2010). In *B. anthracis*, it was demonstrated that the expression of the virulence genes is triggered by bicarbonate (CO_2), resulting in an increased transcription of the *pag* operon as well as higher gene expression of e. g. *eag* (Koehler et al., 1994; Mignot et al., 2003). It was stated, that AtxA might be able to activate further signalling cascades involving additional transcription factors to *pagR* in response to a CO_2 signal (Hoffmaster and Koehler, 1999b). The influence of bicarbonate (CO_2) on the virulence mechanisms and cereulide toxin expression in emetic *B. cereus* should be investigated.

Besides the complex regulation of gene expression behind the toxin synthesis, previous results showed that temperature has an influence on cereulide synthesis at post-transcriptional level (Kranzler et al., 2016). Moreover, temperature, oxygen, bicarbonate, nutrients, the sporulation process, and biofilm formation might influence the cereulide synthesis and its entire regulatory network too (Wijman et al., 2007; Ehling-Schulz et al., 2015; Rouzeau-Szynalski et al., 2020; Dietrich et al., 2021; Huang et al., 2022), which needs to be further characterized in context of the transcriptional regulator PagR. Especially the biofilm formation is very crucial since it was shown that cereulide is able to attach to biofilm complexes and its cereulide production happens in presence of biofilms in emetic *B. cereus* (Huang et al., 2022). Thus, the entire regulatory network of emetic *B. cereus* is a tightly regulated process consisting of a plasmid-chromosome crosstalk, where nutrition, energy, respiration together with intrinsic and extrinsic factors, and the developmental cell status play an important role and where PagR has a significant influence on cereulide toxin synthesis (Ehling-Schulz et al., 2015; Kalbhenn et al., 2022).

4.2 The emetic toxin cereulide and its detection and quantification

The gold standard for cereulide quantification is liquid chromatography coupled with Mass Spectrometry (LC-MS), (Bauer et al., 2010; Biesta-Peters et al., 2010; Stark et al., 2013; Marxen et al., 2015). However, the EN-ISO method (EN ISO 1865) based on Stable Isotope Dilution Assay (SIDA) LC-MS was established for more complex matrices such as cereulide and isocereulides detection in foods, whereas the novel method of Äkta™ pure system coupled with RPC can not be used in complex food matrices because of interference (Bauer et al., 2010; In't Veld et al., 2019; Doellinger et al., 2020; Dietrich et al., 2021; Kalbhenn et al., 2021). Nevertheless, the Äkta™ pure system coupled with RPC is a robust tool, which is easy to establish in laboratories with a focus on biochemistry, microbiology, since the Äkta™ pure system is commonly available and used for purification of proteins or peptides in general (Kalbhenn et al., 2021). An advantage of this newly method is the reproducible and reliable detection, relative quantification, and purification of cereulide by Äkta™ pure system coupled with RPC (Kalbhenn et al., 2021).

Recently published results showed the identification of cereulide producing strains, designated emetic strains, and non-emetic strains, by a routine diagnostic method such as MALDI-TOF MS based on biomarkers underlying a suitable high-throughput technique for microbiological laboratories at universities or industries (Fiedoruk et al., 2016; Ulrich et al., 2019; Doellinger et al., 2020; Dietrich et al., 2021). An advantage of LC-MS and MALDI-TOF MS is that the cross-reactivity is low and the specification for cereulide quantification is high (Ramarao et al., 2020). Another new method was published, where an AlamarBlue-Based Assay was coupled with UPLC-MS/MS (Kranzler et al., 2021).

Isocereulides, namely A-G and H-N, are structure homologs of cereulide and have been reported in contaminated food samples of *B. cereus* (Stark et al., 2013; Marxen et al., 2015; Walser et al., 2022). Cereulide and isocereulide detection and quantification are very important and need to be detected and evaluated in foodborne outbreaks (Walser et al., 2022).

For complex matrices such as food and soil, another recently published method named Artificial Neural Network assisted (ANN) Fourier Transform Infrared (FTIR) Spectroscopy was been developed for the detection of *B. cereus* strains within the *B. cereus* group, especially for the differentiation of strains of the *B. cereus* group such as *Bacillus weihenstephanensis*, *Bacillus cytotoxicus, Bacillus mycoides, Bacillus thuringiensis* as well as *Bacillus cereus*

(Bağcıoğlu et al., 2019). Using the Äkta™ pure system coupled with RPC, the differentiation between *B. anthracis*, *B. thuringiensis,* and various *B. cereus* isolates (emetic strains, emetic-like strains, cereulide deficient isogenic mutants) was possible based on the specific detected cereulide peak at 55 mL and a differentiation between low, medium and high toxin producer could be achieved (Kalbhenn et al., 2021).

Nevertheless, each method for cereulide detection and quantification has its own advantages and disadvantages. This novel tool adapted according to previous research for detection, quantification, and purification of cereulide by RPC can become a valuable, robust, beneficial tool complementing already existing methods and instruments (Kalbhenn et al., 2021).

As the number of foodborne outbreaks and problems with contaminated food increases, reliable and fast methods are needed. However, differentiation between the various strains of the *B. cereus* group and a suitable method for toxin quantification in various matrices is of utmost importance as most *B. anthracis,* many *B. cereus,* and some *B. thuringiensis* pose a major risk to public health.

4.3 *B. thuringiensis, B. cereus* and *B. anthracis,* and their toxins

The recently published results of this PhD thesis showed that biopesticide strains such as *B. thuringiensis* may be detected on foods after harvesting (Johler et al., 2018) and that *B. thuringiensis* is involved in food poising outbreaks with a potential risk for public health (McIntyre et al., 2008; Schwenk et al., 2020; Dietrich et al., 2021).

Indeed, one *B. thuringiensis* strain isolated from rosemary showed high levels of cytotoxicity as well as enterotoxicity compared to the reference, and 74 % of 39 tested isolates exhibited an intermediate level of enterotoxicity, demonstrating the hazardous potential of those strains (Johler et al., 2018). In addition, another study revealed that *B. thuringiensis* biopesticide isolates, foods, and human feces were associated with outbreaks, and all outbreak and food related isolates genomically matched one of six biopesticide strains, leading to the result that a biopesticide product was the origin (Biggel et al., 2022a). It must be emphasized that some *B. cereus*-like strains might also be *B. thuringiensis* as a differentiation of both strains in various food sources is very difficult based on the available methods and techniques leading to uncertainties in the obtained results (EFSA, 2016).

Based on the close relation between the members of the *B. cereus sensu lato* group and the lack of user-friendly and accessible tools, some toxigenic biopesticide strains and other problematic strains of the *B. cereus sensu lato* group cannot be differentiated by routine diagnostics (Helgason et al., 2000; McIntyre et al., 2008; Ehling-Schulz and Messelhäusser, 2012; De Bock et al., 2021). For the differentiation of various strains of *B. cereus sensu lato* group, the U.S. Food and Drug Administration (FDA) published the Bacteriological Analytical Manual (BAM) protocol including microbiological and biochemical assays some years ago (Tallent et al., 2012, 2019; Carroll et al., 2021). A novel method of average nucleotide identity analysis (ANI), ribosomal multi-locus sequence typing (rMLST), and high-resolution melting analysis (HRM) after PCR amplification was developed for the discrimination between *B. thuringiensis* and *B. cereus* (Zhou et al., 2022). Another high-throughput method combining genomics and MALDI-TOF MS analysis based on ribosomal proteins leads to the differentiation of *B. thuringiensis* and *B. cereus* (Chen et al., 2022). New low-cost, user-friendly, rapid, and sensitive approach was developed for the identification of various *B. cereus* strains using biosensors such as cell and phage based immunosensors and DNA biosensors (Ramarao et al., 2020).

Previous studies have shown a low degree of clonality and a high diversity of multi-locus genotypes between *B. cereus* and *B. thuringiensis* suggesting that an exchange of genetic material takes place in their natural environment (Helgason et al., 1998, 2000). A classification was made between the highly pathogenic *B. cereus* group strains and some *B. thuringiensis* biopesticide strains, which are part of another group (De Bock et al., 2021). The pathogenicity of *B. thuringiensis* biopesticide strains cannot be ruled out, whereas the development of specific monitoring tools for the traceability and a better understanding of disease outbreaks related to *B. thuringiensis* and *B. cereus* is needed (McIntyre et al., 2008; Bonis et al., 2021; Biggel et al., 2022b). The biopesticide strain ABTS-1857 (serotype *aizawai*) showed a mid-level of enterotoxin production, a low level of SMase production and was detected in foodborne outbreaks, although this strain has been implicated as the pathogenic agent of a foodborne outbreak in Germany in 2012 (EFSA, 2016; Johler et al., 2018).

Recently, it was suggested that *B. thuringiensis* should be separated into two genomovars designated *B. thuringiensis gv. thuringiensis* or *B. thuringiensis gv. cytolyticus* based on genomic analysis involving insecticidal genes (Baek et al., 2019). The diverse and large family

of insecticidal proteins of e. g. *B. thuringiensis* var. *kurstaki* play an important role in insect control but is on the other hand the major threat due to possible insect resistance (Bravo et al., 2011).

B. cereus may produce emetic toxin cereulide, diarrheal toxins (Hbl, CytK, Nhe), enterotoxin T (BcET), enterotoxin FM (EntFM), hemolysins, phosphatidylcholine phospholipase C (PC-PLC), or sphingomyelinase (SMase) in contrast to *B. anthracis* that produces the anthrax toxin (Bhunia, 2018). Conversely, *B. thuringiensis* produces the enterotoxins Hbl, Nhe, and CytK, as well as SMase and the insecticidal crystal toxins, but cereulide or its biosynthesis genes are not detected (Bhunia, 2018). However, as an example, two psychotolerant *B. weihenstephanensis* strains have been described to produce cereulide at 8 °C and thus may pose a health risk in food products (Thorsen et al., 2006). Moreover, the loss and gain of cereulide synthetase in different members of the *B. cereus sensu lato* group can be observed (Carroll and Wiedmann, 2020). Lately, *B. thuringiensis* isolates, some of which have been linked to biopesticide strains, are involved in an emetic outbreak harboring gene fragments located in the *ces* gene cluster and leading to an exhibited toxicity (Pheepakpraw et al., 2023). Due to the very close genetic relationship of these bacteria and their ability to produce toxins, more attention needs to be paid to develop prevention strategies to minimize outbreaks in the future. Complicating these efforts, taxonomic changes within the *B. cereus* group and the adaption of risk assessment based on specific pathogenicity characteristics and strain-specific virulence, a new paradigm shift was implemented (Biggel et al., 2022b). An summary of the past 25 years of the *B. cereus* group including taxonomic and nomenclatural changes as well as genomic and phenotypic advantages and disadvantages has been given in a recent summary (Carroll et al., 2021).

5. Conclusion and Outlook

This PhD thesis provides the first insights into the role of the novel plasmid-encoded transcriptional regulator PagR influencing the cereulide toxin synthesis in emetic *B. cereus*. As a member of the ArsR/SmtB family, PagR of the emetic *B. cereus* located on a pXO1-like plasmid is a homologue to PagR of *B. anthracis*. The transcriptional regulator PagR binds to the *ces* promoter region *in vitro* and forms dimers that mediate strong protein-DNA interactions. Based on qRT-PCR and UPLC-MS/MS, it was shown that the *ces* expression is elevated during different growth phases, especially in the early (OD_{600} of 0.2 to 7) and late growth phases (24 h, 48 h). Of note, *pagR* of emetic *B. cereus* is transcribed monocistronically in contrast to *pagR* of *B. anthracis,* which is transcribed bicistronically. Future studies should decipher the role of other, novel *pagR*-like transcriptional regulators located on plasmids or the chromosome for a better understanding of the regulatory network in *B. cereus* and *B. anthracis* adding to our understanding related to the One Health concept set forth by the WHO to prevent outbreaks related to foodborne as well as zoonotic pathogens.

Furthermore, a suitable and accessible tool for cereulide detection was adapted using Äkta™ pure system coupled with RPC and validated by canonical UPLC-MS/MS. In the future, this diagnostic tool could be used for a reliable cereulide detection in different mutant strains, as well as be further developed for different matrices for microbiological and biochemical research laboratories all over the world.

A collaboration project with University of Zurich provided important results on the hazardous potential of *B. thuringiensis* in food and foodborne outbreaks. Some members of the *B. cereus* group could not be distinguished by toxin gene profiling, cytotoxicity, FTIR analysis, SplitsTree, *panC* typing, and SMase production due to their high genetic relatedness. Further research should decipher the important mechanisms of enteropathogenicity of *B. cereus* as well as *B. thuringiensis* to improve the individual risk assessment for each microorganism as a relevant foodborne pathogen.

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