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Identification of cereulide producing *Bacillus cereus* by MALDI-TOF MS

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ABSTRACT

The *Bacillus* (*B.*) *cereus* group consists of nine recognized species which are present worldwide. *B. cereus* play an important role in food-borne diseases by producing different toxins. Yet, only a small percentage of *B. cereus* strains are able to produce the heat stable cereulide, the causative agent of emetic food poisoning. To minimize the entry of emetic *B. cereus* into the food chain, food business operators are dependent on efficient and reliable methods enabling the differentiation between emetic and non-emetic strains.

Currently, only time-consuming cell bioassays, molecular methods and tandem mass spectrometry are available for this purpose. Thus, the aim of the present study was to establish a fast and reliable method for the differentiation between emetic/non-emetic strains by MALDI-TOF MS. Selected strains/isolates of the *B. cereus* group as well as other *Bacillus* spp. (total n = 121) were cultured on sheep blood agar for 48 h before analysis.

Subsequently, the cultures were directly analyzed by MALDI-TOF MS without prior extraction steps. The samples were measured in the mass range of *m/z* 800–1800 Da. Using ClinProTools 3.0 statistical software and Flex analysis software (Bruker Daltonics GmbH, Bremen, Germany), a differentiation between emetic/non-emetic isolates was possible with a rate of correct identification of 99.1% by means of the evaluation of two specific biomarkers (*m/z* 1171 and 1187 Da).

1. Introduction

Bacillus (*B.*) species (spp.) are Gram-positive, rod shaped, endospore-forming bacteria occurring world-wide. The *B. cereus* group is genetically highly homogeneous and comprises nine recognized species: *B. anthracis*, *B. cereus* sensu stricto, *B. cytotoxicus, B. mycoides, B. pseudomycoides*, *B. thuringiensis*, *B. toyonensis*, *B. weihenstephanensis* and *B. wiedmannii* (Miller et al., 2018; Vos et al., 2011). Their spores are heat, acid, ultraviolet (UV) and desiccation resistant and survive pasteurization (Bottone, 2010; Clavel et al., 2004).

Due to the persistence of spores in food and the ability of the vegetative cells to produce different toxins, isolates of the *B. cereus* group play an important role in food safety. Several publications report an increasing numbers of cases of foodborne intoxications caused by *B. cereus* (European Centre for Disease Prevention and Control, 2013; Messelhausser et al., 2014). Basically, toxins of the *B. cereus* group can result in two different forms of food intoxication: emetic versus diarrheal (Bottone, 2010).

The diarrheal form is caused by heat-labile proteins, i.e. the two enterotoxin complexes Hbl (hemolytic enterotoxin BL) and Nhe (non-hemolytic enterotoxin) as well as cytotoxin K1 (Schoeni and Wong, 2005; Stenfors Arnesen et al., 2008). In contrast, the emetic form is caused by the small heat-stable cyclic depsipeptide cereulide, encoded by non-ribosomal peptide synthetase genes (*ces*) (Ehling-Schulz, Vukov, et al., 2005). Cereulide without any structural modification has a molecular mass of 1153 Da (Da) and is usually detected as the ammonium adduct ion in LC-ESI-MS measurements (Haggblom et al., 2002). Recently, 18 cereulide variants have been described with molecular masses varying from 1147 to 1205 Da. Interestingly, the cytotoxicity of the different analogues differs widely, for instance, the toxicity of isocereulide A is eight times higher than that of cereulide (Marxen et al., 2005, 2015).

Emetic *B. cereus* strains represent a major hazard in mass catering and are frequently reported as the causative agent of food-borne outbreaks (Ehling-Schulz et al., 2015; European Centre for Disease Prevention and Control, 2013). Cereulide formation in the digestive tract is rarely observed, if it occurs at all. Intoxication is usually caused by the ingestion of toxin preformed in food by *ces*-positive *B. cereus* strains (Ehling-Schulz et al., 2015). The direct identification of toxins in food can be carried out by LC-MS/MS (high performance liquid chromatography-mass

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spectrometry). These methods are able to identify and quantify the toxin with a high sensitivity (Haggblom et al., 2002; In't Veld et al., 2018; Rønning et al., 2015). Generally, food matrices rich in carbohydrates, such as pasta and rice, as well as milk and dairy products are associated with a highest risk of causing cereulide intoxications (Delbrassinne et al., 2012; Messelhausser et al., 2014). Symptoms are mainly characterized by vomiting shortly after ingestion of the toxin and on average cease after one day. Nevertheless, severe cases require hospitalization and several reports of fatal organ failure have been published (Dierick et al., 2005; Mahler et al., 1997; Naranjo et al., 2011; Posfay-Barbe et al., 2008; Tschiedel et al., 2015). In order to be able to take preventive measures in food businesses viable bacteria have to be detected prior to toxin production. Such measures include e.g. the elimination of raw materials contaminated with potentially emetic *B. cereus* strains. For the application of specific hazard control plans, a differentiation between emetic and non-emetic *B. cereus* strains is necessary. However, this requires the availability of a fast and reliable screening method with a high throughput. Emetic and non-emetic *B. cereus* strains cannot be distinguished by standard cultural methods used for isolation (Ehling-Schulz et al., 2015; Stenfors Arnesen et al., 2008). Therefore, mainly molecular methods (Castiaux et al., 2014; Fricker et al., 2007) are applied to differentiate *B. cereus* strains (Ehling-Schulz et al., 2004; Ehling-Schulz et al., 2006; Ehling-Schulz, 2005b). Furthermore, HEp-2/MTS-bioassays can be used for identifying toxic and non-toxic *B. cereus* strains (Beattie and Williams, 1999; Stark et al., 2013). However, these methods are time consuming, difficult and expensive.

For some years MALDI-TOF MS (matrix-assisted laser desorption/ionization time of flight mass spectrometry) is widely used for routine identification of microorganisms (Clark et al., 2013). This method is extremely robust, fast and suited for operation by laboratory personnel without profound knowledge of the technique per se as the results are automatically generated via highly sophisticated statistical software. However, previous attempts to apply this technique to identify emetic *B. cereus* strains were not successful (Fiedoruk et al., 2016). Fiedoruk et al. (2016) described a MALDI-TOF MS approach for the differentiation of emetic and non-emetic *B. cereus* strains by measuring proteins in the mass range of *m/z* 4000–12000 Da. The MALDI-TOF MS method was regarded as a promising and rapid approach for pre-screening of strains, but was not considered an entirely reliable method to distinguish emetic from non-emetic *B. cereus* strains.

The aim of this study was to establish a MALDI-TOF MS method for a reliable differentiation between emetic and non-emetic *B. cereus* strains by directly analyzing the biomass obtained after culturing *B. cereus* on blood-agar. All strains under study were in parallel analyzed by validated molecular (PCR) and bioassay (cell culture) techniques for their ability to produce the emetic cereulide toxin.

2. Material and methods

2.1. Chemicals

Columbia Agar with 5% sheep blood was purchased from VWR (Darmstadt, Germany). The MALDI-TOF MS matrix CHCA (α-cyano-4-hydroxycinnamic acid), aqua dest. and acetonitrile were obtained from Fluka (Fluka, Dagebóll, Germany). Trifluoracetic acid (TFA) and hydrochloric acid were obtained from Merck (Merck, Hamburg, Germany). Synthetic cereulide standard purchased from Chiralix (Netherlands) was dissolved in ethanol at a concentration of 1 mg/ml and was used to determine the detection limit of the applied MALDI-TOF MS approach.

The matrix for MALDI-TOF MS was prepared according to Meetani and Voorhees (2005) by dissolving 10 mg CHCA in 1 ml organic solvent (700 µl acetonitrile, 300 µl aqua dest., 1 µl TFA).

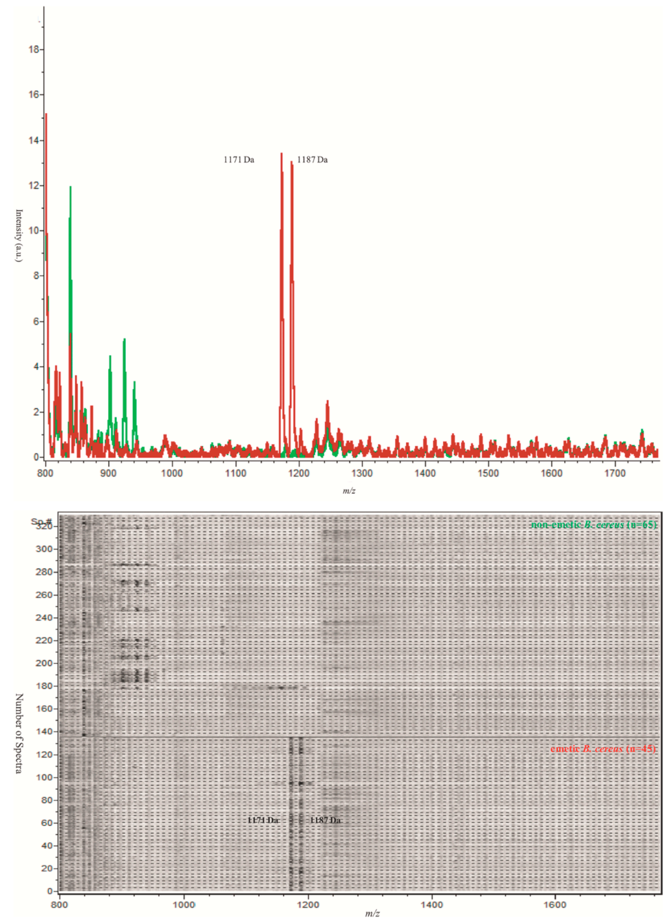

Fig. 1. Profile of the analyzed *B. cereus* strains/isolates shown as sum spectra (Fig. 1a; emetic strains in red, non-emetic strains in green) and in band view (Fig. 1b).

2.2. Bacterial strains and culture conditions

In total, 117 *B. cereus* sensu lato strains/isolates and 4 other *Bacillus* spp., commonly found in food, were examined and differentiated with respect to their toxic potential (cereulide production). The *B. cereus* sensu lato set comprised 100 isolates (see Table 2), 13 reference strains (see Tables 3) and 4 low-producing emetic isolates (see Table 4). Reference strains were purchased from DSM (German Collection of Microorganisms and Cell Cultures), analyzed isolates were part of the culture collection of the Chair for Hygiene and Technology of Milk (MHI, Department of Veterinary Sciences, LMU Munich). Isolates and strains under study were stored before use at −80 °C by using the MAST CryobankTM system for long-term preservation of bacteria. Emetic *B. weihenstephanensis* (MC 118) were provided by the Weihenstephan *Bacillus cereus* collection (TU Munich). All *B. cereus* strains were analyzed by molecular methods (PCR assay) and bioassays (HEp-2 cytotoxicity test) for their potential to produce the emetic toxin according to the methodology described in previous studies (Wehrle et al., 2009, 2010). In brief, isolates under study were enriched in skimmed milk medium (SMM) for 18 h at 32 °C, and, after autoclaving, analyzed for cereulide production by a cytotoxicity test based on HEp-2 cells as described by Ehling-Schulz (2005b). PCR analyses have been described in detail by Wehrle et al. (2009), CesF1 and CesR2 was used as primer pair. For the MALDI-TOF MS measurements, strains/isolates under study were

Table 1
ClinProTools Peak Statistic for the selected mass peaks used for differentiation of non-emetic (class 1) and emetic (class 2) *B. cereus* strains.

Mass Peak	p-value *t*-test/ ANOVA	Average intensityclass 1 (a. u.)	Average intensityclass 2 (a. u.)	Standard Deviation class 1 (%)	Standard Deviation class 2 (%)	Receiver Operating Characteristic (AUC)
1171	< 0.05	1.26	6.6	0.53	2.96	0.99
1187	< 0.05	1.11	11.32	0.33	6.72	1.00

a.u.: arbitrary units; AUC: area under curve.

cultured for 48 h at 37 °C on Columbia Agar with 5% sheep blood (pH 7.3 ± 0.2).

2.3. Sample preparation

A small amount (one tip of a wooden application stick) of one colony was transferred directly from the culture medium onto a ground steel target (MTP 384 target plate ground steel BC, Bruker Daltonics GmbH, Bremen, Germany). The spot was overlaid with 1 µl matrix (CHCA) and air-dried at room temperature (approx. 22 °C). After the spot was dried, the sample was again overlaid with 1 µl matrix (CHCA) and allowed to air-dry at room temperature.

2.4. MALDI-TOF MS measurements and data processing

An Autoflex Speed MALDI-TOF/TOF MS (Bruker Daltonics GmbH, Bremen, Germany) was used for measurements which were performed in linear positive mode (*m/z* 800–1800 Da).

The following parameters were set: random walk of partial sample with ten shots at a raster spot with a limit diameter of 2000 µm; sample rate and digitizer settings were set to 2.00 GS/s, the smartbeam laser was set to "flat" with a frequency of 1000.0 Hz. For automatic measurement an "AutoX" method was created using the "flex control" software. Basic laser settings were laser energy 68–78% (global attenuator offset 24%) with the following high voltage settings: ion source 1, 19.50 kV; ion source 2, 18.2 kV; lens, 7.0 kV; pulsed ion extraction set to 340 ns. In total, 1000 single spectra per 200 shots were accumulated.

The Bacterial Test Standard (BTS) from Bruker Daltonics GmbH (mass range: 3637.8–16,952.3 Da) was used as calibration standard on a regular basis once a week. During each measurement the peptide standard II from Bruker Daltonics GmbH (mass range: 700–3500 Da) was used for calibration.

All isolates were cultured twice on different days (biological replicates, n = 2) and each eight technical replicates were measured per biological replicate. For the differentiation of the emetic and non-emetic strains, the Genetic Algorithm of the statistical analysis software ClinProTools 3.0 (Bruker Daltonics GmbH, Bremen) was applied with the following settings: maximum of peaks: 4; maximum number of generations: 40; mutation rate: 0.2; crossover rate: 0.5. Statistical models were developed with ten non-emetic (class 1, strains no. 86, 1475-9 and 1481-4) and ten emetic (class 2, strains no. 280, 1305, 1326, 1471, 1485-7, 1515, 1517 and 1528) isolates (n = 20, 160 spectra). For the external validation according to the software, five emetic (strains no. 1533-5, 1538 and 1542) and five non-emetic strains (strains no. 1489-93) (n = 10, n = 80 mass spectra) were processed. All other isolates were used for classification (see Table 2). For additional quality control all spectra were visually checked for differences in mass peaks with the Flex analysis software (Bruker Daltonics GmbH).

To evaluate the sensitivity of the applied MALDI-TOF MS technique a commercially available synthetic cereulide standard was analyzed at varying concentrations. For this purpose, the toxin standard was spotted directly onto the target, air dried and overlaid with the CHCA matrix. The standard was measured in concentrations of 0.001–10 µg/ml.

3. Results

In a preliminary test the mass spectra of twenty *B. cereus* strains/isolates (ten emetic and non-emetic isolates each) grown on blood agar for 48 h were analyzed by MALDI-TOF MS. The processing of the mass spectra was performed via statistical models of the ClinProTools 3.0 software. The obtained results indicated the basic applicability of the method for the differentiation of emetic and non-emetic strains (results not shown). Overall, each mass spectrum consisted of approximately 38 discernible mass peaks. Two mass peaks which consistently appeared in all mass spectra of the emetic strains had prominent intensity differences suitable for the differentiation (*m/z* [mass per charge] 1171 and 1187 Da). The emetic strains clearly showed these two mass peaks in their mass spectra, whereas these mass peaks were not detectable within spectra of the non-emetic strains (Fig. 1a and b).

To further prove the general applicability of the developed method, a broad range of *B. cereus* isolates including an emetic *B. weihenstephanensis* previously described by Thorsen et al. (2006) and 17 *Bacillus* spp. reference strains (Table 3) were analyzed (Guerin et al., 2017). These analyses were performed as a blind study, i.e. the MALDI-TOF MS experimenter was ignorant of the assignment of the isolates. Results summarized in Table 2 (*B. cereus* isolates) and Table 3 (reference strains) corroborated the findings of the preliminary study. Moreover, reproducibility of the analyses is demonstrated by the fact that the *m/z*-variances of biological and technological replicates for emetic strains were below 1 Da. The variances in intensity of biological and technological replicates varied from 0.1 to 0.3 arbitrary units (a.u.) for emetic strains.

Significant differences ($p < 0.05$) between the average peak intensities of the two different data sets (non-emetic class 1 and emetic class 2) were obtained by *t*-test/ANOVA and the Receiver Operating Characteristic (ROC) values (Table 1). These differences allowed the reliable identification of emetic strains. The mean mass peak intensities ranged from 1.11 to 11.32 a.u. The ROC values varied from 0.99 to 1.00 AUC (Area Under Curve). Both mass peaks were included in the differentiation model as significant differences in mass peak intensities were evident between emetic and non-emetic strains. The difference in average intensities was 5.34 for the *m/z* 1171 Da mass peak and 10.21 for the *m/z* 1187 Da mass peak. The relative standard deviation for the mass peak intensities varied from 0.33 to 6.72% (Table 1).

Overall, the statistical model based on the average mass peak intensity differences of the above-mentioned mass peaks enabled the reliable differentiation between emetic (42) and non-emetic (75) strains, including 17 reference strains (Tables 2 and 3). All mass spectra were also evaluated visually using the software "flex analysis" (Bruker Daltonics GmbH)in order to detect the above-mentioned mass peaks. Altogether, a consistency of 100% of the MALDI-TOF MS results with the results of characterization of the strains via bioassay and PCR was achieved.

As several previous studies have pointed out a great variability in the toxin productivity of emetic *B. cereus* strains, we additionally analyzed four low-producing *B. cereus* isolates (Table 4). Apart from the isolate IH 41385 which is well-known for its extremely low productivity, probably caused by a point mutation in the *ces* gene (Stark et al., 2013), all other analyzed low-producing isolates were correctly

Table 2
Differentiation between emetic (+) and non-emetic (−) *B. cereus* isolates by MALDI-TOF MS. Classification of the isolates under study was based on the results of bioassay (cytotoxicity assay) and PCR analyses. Isolates used for the development of statistical models and the external validation are marked with "s" and "v", respectively.

No. (MHI)	Origin	MALDI-TOF MS[a]	Bioassay/PCR[b]
86[s]	FI	–	−/−
280[s]	FI	+	+/+
1305[s]	FI	+	+/+
1326[s]	HI	+	+/+
1471[s]	FI	+	+/+
1475[s]	FI	–	−/−
1476[s]	FI	–	−/−
1477[s]	FI	–	−/−
1478[s]	FI	–	−/−
1479[s]	FI	–	−/−
1481[s]	FI	–	−/−
1482[s]	FI	–	−/−
1483[s]	FI	–	−/−
1484[s]	EI	–	−/−
1485[s]	FI	+	+/+
1486[s]	FI	+	+/+
1487[s]	HI	+	+/+
1489[v]	FI	–	−/−
1490[v]	FI	–	−/−
1491[v]	FI	–	−/−
1492[v]	FI	–	−/−
1493[v]	FI	–	−/−
1494	FI	–	−/−
1495	FI	–	−/−
1496	FI	–	−/−
1497	FI	–	−/−
1499	FI	–	−/−
1500	FI	–	−/−
1501	FI	–	−/−
1502	FI	–	−/−
1503	FI	–	−/−
1508	FI	–	−/−
1509	FI	–	−/−
1510	FI	–	−/−
1511	FI	–	−/−
1512	FI	–	−/−
1513	FI	–	−/−
1514	FI	–	−/−
1515[s]	FI	+	+/+
1516	FI	–	−/−
1517[s]	FI	+	+/+
1518	FI	–	−/−
1519	FI	–	−/−
1520	FI	–	−/−
1521	FI	–	−/−
1522	FI	–	−/−
1523	FI	–	−/−
1524	FI	–	−/−
1525	FI	–	−/−
1526	FI	–	−/−
1527	FI	–	−/−
1528[s]	FI	+	+/+
1529	FI	–	−/−
1530	FI	–	−/−
1531	FI	–	−/−
1532	FI	–	−/−
1533[v]	FI	+	+/+
1534[v]	FI	+	+/+
1535[v]	FI	+	+/+
1538[v]	FI	+	+/+
1542[v]	FI	+	+/+
1548	HI	+	+/+
1549	FI	+	+/+
1550	HI	+	+/+
1553	FI	+	+/+
1559	FI	+	+/+
1562	HI	+	+/+
1566	HI	+	+/+
1567	HI	+	+/+
1568	HI	-	−/−
1569	HI	+	+/+
1571	HI	+	+/+
1654	EI	+	+/+
1664	FI	+	+/+
1665	FI	+	+/+
1670	FI	–	−/−
1672	FI	+	+/+
1673	FI	+	+/+
1678	FI	–	−/−
1699	FI	+	+/+
1701	HI	+	+/+
1745	FI	+	+/+
1885	EI	–	−/−
2011	HI	+	+/+
2049	HI	+	+/+
2058	HI	+	+/+
2350	FI	–	−/−
2507	FI	–	−/−
2572	FI	+	+/+
3032	FI	+	+/+
3104	FI	–	−/−
3108	FI	–	−/−
3161	HI	–	−/−
3168	FI	+	+/+
3178	HI	–	−/−
3185	HI	–	−/−
3236	FI	+	+/+
3322	FI	–	−/−
B. weihenstephanensis			
MC 118[c]	EI	+	+/+
MHI 184	EI	–	−/−

-: non-emetic strains +: emetic strains; FI: food isolate; HI: human isolate; EI: environmental isolate.

[a] Biomarkers (*m/z* 1171 and 1187 Da) detectable (+) or not detectable (−).

[b] Isolates marked with "s" and "v" as well as *B. weihenstephanensis* were reanalyzed for this study as described in the materials/methods section. For all other isolates data are from Wehrle et al. (2009) or were analyzed in our lab in other unpublished studies.

[c] Originally described as emetic *B. weihenstephanensis* (Thorsen et al., 2006); obtained from the WSBC Weihenstephan *Bacillus cereus* collection, ZIELWeihenstephan, TU Munich (Germany).

Table 3
Analyses of *Bacillus* spp. type ([T]) and other reference strains by MALDI-TOF MS and cereulide specific bioassays/PCR.

Strain	Species	MALDI-TOF MS[a]	Bioassay/PCR
B. cereus sensu stricto			
DSM 31[T]	*B. cereus*	–	−/−
DSM 2301	*B. cereus*	–	−/−
DSM 4222	*B. cereus*	–	−/−
DSM 4282	*B. cereus*	–	−/−
DSM 4312	*B. cereus*	+	+/+
DSM 4384	*B. cereus*	–	−/−
DSM 8438	*B. cereus*	–	−/−
B. cereus group			
DSM 22905[T]	*B. cytotoxicus*	–	−/−
DSM 2048[T]	*B. mycoides*	–	−/−
DSM 12442[T]	*B. pseudomycoides*	–	−/−
DSM 2046[T]	*B. thuringiensis*	–	−/−
DSM 11821[T]	*B. weihenstephanensis*	–	−/−
DSM 102050[T]	*B. wiedmannii*	–	−/−
Other *Bacillus* spp.			
DSM 7[T]	*B. amyloliquefaciens*	–	−/−
DSM 10[T]	*B. subtilis*	–	−/−
DSM 13[T]	*B. licheniformis*	–	−/−
DSM 27[T]	*B. pumilus*	–	−/−

-: non-emetic strains +: emetic strains.

[a] Biomarkers (*m/z* 1171 and 1187 Da) detectable (+) or not detectable (−).

Table 4
Toxin productivity of low-producing *B. cereus* isolates according to previous publications and in-house data.

B. cereus	Toxin productivity (ng cereulide/mg biomass fresh weight)[a]	MS signal intensity (a.u.)[b]	Cytotoxic activity(reciprocal titer)[c]	MALDI-TOF results[d]
Isolates				
IH 41385	0.5–1	traces	< 10	negative
RIVM BC379	7–9	< 50	76	positive
UHDAM B315	50–90	> 200	143	positive
RIVM BC51	n.d.	< 50	> 1000	positive
Control strains				
DSM 4312 (F 4810/72)	240–600	> 150	> 1000	positive
MHI 1305	170–200	> 75	230	positive

a.u.: arbitrary units; n.d.: not detected.
[a] Data are from Carlin et al. (2006); in this study *B. cereus* was grown on TSA plates and biomass was extracted with methanol.
[b] Data are from Stark et al. (2013); in this study *B. cereus* was enriched in LB broth, and cell pellets were extracted with ethanol.
[c] In-house data; *B. cereus* was enriched in skimmed milk medium. The autoclaved broth was analyzed by a cytotoxicity assay based on HEp-2 cells.
[d] Biomarkers (m/z 1171 and 1187 Da) detectable (positive) or not detectable (negative).

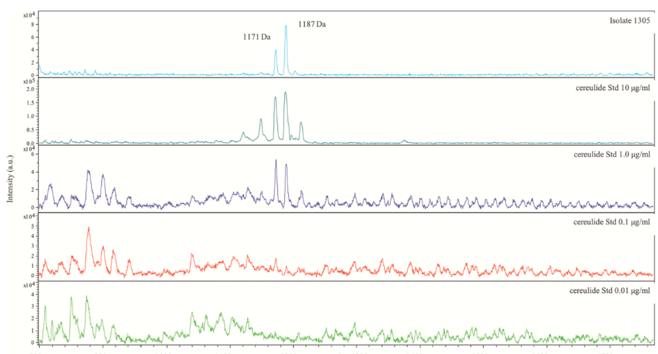

Fig. 2. Measurement of synthetic cereulide standard in different concentrations compared to an emetic *B. cereus* strain (strain no. 1305).

identified as emetic *B. cereus* strains by applying the established MALDI-TOF MS technique (Table 4). The high sensitivity of the developed method was also confirmed by the analyses of a synthetic cereulide standard. Measuring different concentrations of this synthetic preparation, the detection limit was 1.0 µg/ml when following the described sample preparation protocol.

4. Discussion

Bacillus cereus is abundant in the environment and is therefore frequently found in food. Low levels of *B. cereus* cells or spores are present on virtually every raw agricultural commodity. Particularly herbs and other food items with direct soil contact are at high risk for contamination with *B. cereus* (Ceuppens et al., 2013; Stenfors Arnesen et al., 2008). Due to their food poisoning potential higher levels of *B. cereus* in food constitute a public health hazard and represent a major problem for the food industry. This applies particularly to the emetic strains capable of producing high amounts of the heat-stable cereulide in food. Depending on the food category investigated, prevalence rates for emetic strains show a broad variability. Percentages in the range from < 1% in vegetables to > 20% in farinaceous products have been reported (Biesta-Peters et al., 2016; Delbrassinne et al., 2012; Ehling-Schulz et al., 2015). To improve HACCP (hazard analysis and critical control points) based concepts and to prevent foodborne intoxications by emetic *B. cereus* it is necessary to identify the contamination sources in the food production chain (Messelhausser et al., 2014). This requires novel diagnostic strategies as morphological or microscopic approaches are nearly useless for the differentiation of emetic and non-emetic *B. cereus* strains (Ehling-Schulzet al., 2005a, b). Identification of emetic strains is currently only possible by complex and sophisticated methods such as PCR, bioassays or mass spectrometry (Ehling-Schulz et al., 2004; Fiedoruk et al., 2016; Messelhausser et al., 2014; Rønning et al., 2015; Stark et al., 2013).

In contrary, MALDI-TOF MS can be used as a fast screening method for routine microbiological analysis since minimal sample pretreatment is required. Therefore, this technique appears to be the method of choice for the differentiation of emetic and non-emetic strains. However, up to now, only one study has been published in which the applicability of this technique to this purpose was evaluated (Fiedoruk et al., 2016). In principle, the authors used an indirect approach by measuring differences in the mass spectra profiles in positive linear ionization mode in the range of *m/z* 4000–12,000 Da. Ultimately, after analyzing more than 100 *B. cereus* isolates, the authors stated that proteomic profiling of whole cells by MALDI-TOF MS is not a sufficiently reliable method to distinguish emetic and non-emetic *B. cereus* isolates. The differentiation of *Bacillus* spp. within the *Bacillus cereus* sensu lato group is not possible via MALDI-TOF MS using commercial databases only (Fernández-No et al., 2013).

Therefore, in the present study a direct approach to differentiate between emetic and non-emetic strains was chosen. Obviously, the direct approach has the limitation of being dependent on the production of cereulide on the culture medium and the temperature used for cultivation. If a strain has the ability to produce cereulide during culture conditions used for pre-culture, the result of the MALDI-TOF MS measurement would be false negative. In principle, like previously described for many other bacterial toxins, cereulide production depends strongly on the growth and enrichment conditions applied (Ehling-Schulz et al., 2015; Kranzler et al., 2016). For the evaluation of the toxin productivity of emetic *B. cereus* strains, in previous studies incubation on tryptic soy agar (TSA) for up to ten days at 28 °C was followed by extraction with organic solvents (Andersson et al., 1998). A more rapid approach was used by Stark et al. (2013) in which isolates were precultured in LB (lysogeny) broth (TSB, tryptic soy broth) and then enriched overnight at 24 °C. However, the subsequent extraction of the cell pellet took up to 17 h. While both culture media resulted in high toxin amounts, no toxin productivity could be observed in other media such as BHI (brain heart infusion) and peptone broth commonly used for the enrichment of bacteria (Finlay et al., 2000). Less known is the fact that blood agar also represents an excellent medium for cereulide production (Jääskeläinen et al., 2003). Coincidentally, culturing bacteria on blood agar at 37 °C is also recommended by Bruker Daltonics for the identification of bacteria by MALDI-TOF MS (Bruker Daltonics, 2014). Thus, it seemed possible to develop a simple and fast analysis method for the differentiation of emetic and non-emetic *B. cereus* isolates. The analysis of more than 100 isolates confirmed this assumption impressively. The similarity of the mass peaks (*m/z* 1171 and 1187 Da, Fig. 1) found after analyzing whole cells of emetic strains with the detected mass peaks of the cereulide standard (Fig. 2), clearly indicates that these mass peaks represent the cereulide produced by the strains on blood agar. Moreover, as bacterial cultures were directly measured from blood agar plates, analyses were completed within 5 min.

Overall, the method worked very well, 120 out of 121 tested strains/isolates including one emetic *B. weihenstephanensis* were correctly identified. Only one emetic strain (IH 41385, Table 4) reacted false negative in the MALDI-TOF MS. This particular strain is well-known for its extremely low productivity (Carlin et al., 2006; Stark et al., 2013). Considering the toxin dose needed to induce emesis, i.e. 8 μg cereulide per kg body weight (Isobe et al., 1995), it is highly unlikely that such low-producer strains represent a public health hazard.

In conclusion, the developed method is characterized by a high inclusivity of > 99% and a striking simplicity. Theoretically, a strain may produce cereulide in a food matrix but not on a blood agar (Rajkovic et al., 2006). However, in our analyses including a comprehensive range of emetic *B. cereus* strains from different sources we found no indication for this scenario. If a modified version of the method is also applicable to the detection of other enterotoxins, i.e. hemolysin BL, nonhemolytic enterotoxin and cytotoxin K, produced by enterotoxigenic diarrheal *B. cereus,* will be subject of future studies.

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