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Assessing the toxic potential of enteropathogenic *Bacillus cereus*

Nadja Jessberger[a,*,1], Markus Kranzler[b,1], Claudia Da Riol[a], Valerie Schwenk[a], Tanja Buchacher[b,2], Richard Dietrich[a], Monika Ehling-Schulz[b], Erwin Märtlbauer[a]

[a] Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-Universität München, Schönleutnerstr. 8, 85764, Oberschleißheim, Germany
[b] Functional Microbiology, Institute of Microbiology, Department of Pathobiology, University of Veterinary Medicine Vienna, 1210, Vienna, Austria

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ABSTRACT

The diarrheal type of food poisoning caused by enteropathogenic *Bacillus cereus* has been linked to various exotoxins. Best described are the non-hemolytic enterotoxin (Nhe), hemolysin BL (Hbl), and cytotoxin K (CytK). Due to the ubiquitous prevalence of *B. cereus* in soil and crops and its ability to form highly resistant endospores, contaminations during food production and processing cannot be completely avoided. Although phylogenetically closely related, enteropathogenic *B. cereus* strains show a high versatility of their toxic potential. Thus, functional tools for evaluating the pathogenic potential are urgently needed in order to predict hazardous food contaminations. As the diarrheal syndrome is the result of a toxico-infection with enterotoxin production in the intestine, the entire passage of the bacteria within the host, from spore survival in the stomach, spore germination, host cell adherence, and motility, to enterotoxin production under simulated intestinal conditions was compared in a panel of 20 strains, including high pathogenic as well as apathogenic ones. This approach resulted in an overarching virulence analysis scheme. In parallel, we searched for potential toxico-specific secreted markers to discriminate low and high pathogenic strains. To this end, we targeted known exotoxins using an easy to implement immunoblotting approach as well as a caseinolytic exoprotease activity assay. Overall, Nhe component B, sphingomyelinase, and exoproteases showed good correlation with the complex virulence analysis scheme and can serve as a template for future fast and easy risk assessment tools to be implemented in routine diagnostic procedures and HACCP studies.

1. Introduction

Bacillus cereus is an important pathogen involved in food poisoning outbreaks (Mead et al., 1999; Scallan et al., 2011; Anonymous, 2015; Messelhäußer and Ehling-Schulz, 2018). Although the course of disease is often mild and self-limiting, severe and even lethal cases are published (Lund et al., 2000; Dierick et al., 2005; Naranjo et al., 2011). Two types of gastrointestinal diseases, the emetic and the diarrheal syndrome, are known (Stenfors Arnesen et al., 2008; Ehling-Schulz et al., 2019). The emetic form, which manifests in vomiting and nausea, is caused by the heat-stable cyclic dodecadepsipeptide cereulide (Agata et al., 1995; Ehling-Schulz et al., 2004; Messelhäußer et al., 2014). Cereulide is pre-formed in contaminated foods and thus, food intoxication appears shortly after ingestion. Diagnostics of emetic *B. cereus* has been massively improved by developing PCR methods for the detection of emetic strains as well as mass-spectrometry for cereulide quantification in foods (Ehling-Schulz and Messelhäußer, 2013; Messelhäußer et al., 2014; Ehling-Schulz et al., 2015). Recently, the identification of cereulide producing *B. cereus* by MALDI-TOF MS was described (Ulrich et al., 2019). These methods contribute to an easier and more reliable discrimination between hazardous and harmless food samples. Unlike the emetic toxin, the enterotoxins responsible for the diarrheal form are heat-, protease- and acid labile (Stenfors Arnesen et al., 2008). This means that enterotoxins pre-formed in foods do most certainly not contribute to the disease, but rather the enterotoxins produced in the intestine. Three enterotoxins have been linked to the diarrheal form of disease, which are the single protein CytK (cytotoxin K (Lund et al., 2000);) and mainly the two three-component toxin complexes Nhe (non-hemolytic enterotoxin (Lund and Granum, 1996; Didier et al., 2016);) and Hbl (hemolysin BL (Beecher and MacMillan, 1990; Beecher et al., 1995);). Nhe consists of the proteins NheA, NheB and NheC, while Hbl comprises the two lytic proteins L1 and L2 and the binding component B. Tools for the detection of these enterotoxins in culture supernatants of *B. cereus* are available, which are based on

* Corresponding author.
E-mail address: N.Jessberger@mh.vetmed.uni-muenchen.de (N. Jessberger).
[1] contributed equally.
[2] Present address: Turku Center for Biotechnology, University of Turku and Åbo University, Tykistökatu 6, 20520 Turku, Finland.

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monoclonal antibodies against the single toxin components (Dietrich et al., 1999, 2005; Tausch et al., 2017). In addition to these toxins, several other virulence factors have been described that might also contribute to enteropathogenicity. These virulence factors include enzymes such as the sphingomyelinase (SMase) and the phosphocholine-specific phospholipase C (PC-PLC), as well as proteinases such as immune inhibitor A metalloproteases (InhAs) and collagenase-specific matrix metalloproteases such as ColA (for review see Ehling-Schulz et al., 2019). However, for a holistic virulence assessment of enteropathogenic *B. cereus*, only investigating their ability to produce toxins is insufficient, as the establishment of a *B. cereus*-associated food infection also depends on several other factors. First of all, the ability of the bacteria, mainly spores, to survive the stomach passage (Clavel et al., 2004; Wijnands et al., 2009; Ceuppens et al., 2012; Berthold-Pluta et al., 2015) is of utmost importance. Only spores able to germinate will contribute to the onset of disease (Clavel et al., 2004; Wijnands et al., 2006, 2007). Furthermore, adherence to the intestinal epithelium, which helps the bacteria to settle in the intestine, as well as the ability to actively move towards the epithelium, can play a decisive role in the course of disease (Minnaard et al., 2004; Ramarao and Lereclus, 2006; Ghelardi et al., 2007; Salvetti et al., 2007; Mazzantini et al., 2016). Thus, for the prediction of the health risk originating from a certain enteropathogenic *B. cereus* isolate, the aforementioned parameters have to be considered. A strain, for instance, which produces high levels of toxins but its spores are not able to germinate in the host environment could be classified less hazardous than a lower toxin producer with high stomach survival rates. In an animal model, all these parameters could be addressed simultaneously, but currently no suitable animal model exists for *B. cereus*. Hence, the aim of this study was to create an overarching virulence assessment scheme for enteropathogenic *B. cereus*, considering parameters from bacterial survival during stomach passage to enterotoxin production under simulated intestinal conditions. To achieve this, the pathogenicity parameters mentioned above were compared in a set of 20 *B. cereus* strains, including high toxic as well as apathogenic isolates. However, since such in depth investigations are not suitable for fast and easy virulence assessment in routine diagnostics of food samples, we developed an easy to implement analysis tool, based on secreted bacterial virulence factors, which reflected the results of the detailed virulence assessment.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *B. cereus* strains used in this study are listed in Table 1. Additionally, strains 2/25, 1/26, 1/27, 1/28, 1/30, 11/6 II AR, 10/12 D from two food poisoning outbreaks in Austria (Schmid et al., 2016) were examined. Strains were pre-cultured in 20 ml CGY (casein-glucose-yeast) medium with 1% glucose at 37 °C and 120 rpm for 17 h. For motility assays, OD_{600} of all strains was adjusted to 20. To adjust the MOI in adhesion assays, cfu of all strains at OD_{600} = 0.05 was determined. For this, dilution series of 10^{-3} - 10^{-7} were applied to CGY agar plates. For incubation under simulated intestinal conditions, preparation of cRPMI medium was carried out as described previously (Jessberger et al., 2017). In brief, RPMI 1640 medium (with stable glutamine; Biochrom GmbH, Germany), supplemented with 1% glucose and 2% casein hydrolysate, was incubated with differentiated CaCo-2 cells for 22 h and filtered using a 0.2 µm filter. The pre-culture was adjusted to a start OD_{600} of 0.05 in 45 ml cRPMI and incubated statically at 37 °C and 7% CO_2 atmosphere. Samples for analyses were taken after 2, 3, 4, and 6 h, and centrifuged at 3500 g at 4 °C for 15 min. For immunoblot and protease activity analyses, the supernatant was filtered through a 0.2 µm Filtropur filter (Sarstedt, Germany) and stored at −80 °C until use.

2.2. Cell lines and culture conditions

CaCo-2 cells were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and cultivated in RPMI 1640 medium (with stable glutamine) supplemented with 10% fetal bovine serum (Biochrom AG). For cultivation, 80 cm^2 culture flasks and a humidified incubator (37 °C and 7% CO_2) were used. Cells were passaged 1:6 every 3–4 d. For adhesion assays, 5×10^4 CaCo-2 cells/well were seeded in 24 well plates and differentiated for 14 d. Medium was changed every 2–3 d. According to Jessberger et al. (2017), differentiated CaCo-2 cells were incubated for 22 h with RPMI 1640 medium containing 1% glucose and 2% casein hydrolysate to gain "CaCo-2 treated" (cRPMI) medium.

2.3. Spore preparation

B. cereus spores were prepared as described previously (Fricker et al., 2011; Da Riol et al., 2018). Briefly, cultures were incubated for 3 d in minimal sporulation medium (1 M $MgCl_2$ x 6 H_2O, 1M Ca $(NO_3)_2 \times 4\ H_2O$, 12.5 mM $ZnCl_2$, 2.5 mM $CuCl_2$, 2.5 mM $CoCl_2$ x 6 H_2O, 2.5 mM Na_2MoO_4 x 2 H_2O, 5 mM $(NH_4)_2SO_4$, 0.066 mM $MnSO_4$ x H_2O, 1 mM $FeSO_4$ and 1 M maltose in bacto nutrient broth (Oxoid); pH 7.6) until > 80% spore content was reached. Sporulation was monitored via bright-field microscopy. After centrifugation at 4000 g, 7 min and 4 °C, the cultures were washed three times in pre-cooled spore washing buffer (2 mM KH_2PO_4, 8 mM K_2HPO_4, pH 7.4) and stored at 4 °C. To determine spore concentrations, washed spore suspensions were again centrifuged (4000 g, 7 min, 4 °C) and resuspended in 5 ml spore washing buffer. OD_{600} was adjusted to 0.4.4 × 200 µl were transferred to Eppendorf tubes. 2 × 200 µl were heated for 10 min at 80 °C. Subsequently, a 1:10 dilution series of all samples was prepared and plated on CGY agar. Heat treatment guaranteed the determination of the pure spore count by eliminating residual vegetative bacteria. Furthermore, the comparison of heat treated and untreated samples allowed verifying the purity of the spore preparations. For cfu determination, the weighted arithmetic mean of dilutions 10^{-2} to 10^{-4} was used.

2.4. Adhesion to CaCo-2 cells

Adhesion assays were performed with vegetative cells and spores of 20 *B. cereus* strains. Overnight cultures as well as spore suspensions were adjusted to the desired MOI in RPMI 1640 medium. Differentiated CaCo-2 cells were washed twice with 1 ml PBS and infected with 1 ml bacterial suspension. MOI 1 and 10 were used for vegetative cells, MOI 1 for spores. After infection, samples were incubated at 37 °C and 7% CO_2 for 30 min. The bacterial suspension was siphoned, and cells were washed three times in 1 ml PBS and lysed in 1 ml H_2O. Spore suspensions were incubated at 80 °C for 10 min to eliminate putative residual or newly germinated vegetative cells. Serial dilutions were plated on CGY agar. After 24 h incubation at 37 °C, cfu were determined and the number of adhered bacteria or spores was calculated in relation to the inoculum. Each strain was tested in triplicates.

2.5. Germination

Germination of *B. cereus* spores was compared in CGY, RPMI and cRPMI medium, each with and without prior heat activation for 10 min at 80 °C. As described before (Jessberger et al., 2019), media were inoculated to an OD_{600} = 1. Samples (200 µl, 3 technical replicates each) were incubated at 37 °C for 1 h in 96-well plates. Every 3 min the optical density at 620 nm was measured in a Tecan photometer using Ridawin software. At least 2 replicates per strain were examined. Germination is shown as the decrease of OD_{620} (in %) per time.

Table 1
B. cereus strains used in this study. A set of 19 enteropathogenic and apathogenic strains has already been characterized in detail (Jessberger et al., 2015, 2017). Probiotic IP 5832 (Kniehl et al., 2003) was added to the analyses.

B. cereus strain	Origin	Genotype Clade (group)	Toxin gene profiling *ces hbl nhe cytK2* profile					Pathogenicity*
14294-3 (M6)	Ice cream	I (III)	–	+	+	+	A	m/hi
SDA KA96	Raw milk	I (III)	–	+	+	+	A	hi
INRA A3	Starch	II (IV)	–	+	+	+	A	m/lo
INRA C3	Pasteurized carrot	II (IV)	–	+	+	+	A	hi
6/27/S	Human feces	II (IV)	–	+	+	+	A	m
F3175/03 (D7)	Human feces	II (IV)	–	+	+	+	A	m
RIVM BC 934	Lettuce	II (IV)	–	+	+	+	A	lo
F528/94	Beef & chow mein and rice, outbreak	I (II)	–	+	+	–	C	lo
F837/76	Human, postoperative infection	I (III)	–	+	+	–	C	hi
RIVM BC 126	Human feces	I (II)	–	+	+	–	C	hi
MHI86	Infant food	I (III)	–	–	+	+	D	lo
F4429/71	Vanilla pudding	I (III)	–	–	+	+	D	hi
RIVM BC 964	Kebab	II (IV)	–	–	+	+	D	hi
F3162/03 (D8)	Human feces	I (III)	–	–	+	+	D	m/hi
MHI226	Milk and milk products	I (III)	–	–	+	–	F	lo
NVH 0075-95	Stew with vegetables, foodpoisoning	I (III)	–	–	+	–	F	hi
WSBC10035	Pasteurized milk	I (III)	–	–	+	–	F	m/hi
RIVM BC 90	Human feces	I (III)	–	–	+	–	F	lo
7/27/S	Human feces	I (III)	–	–	+	–	F	m/hi
IP 5832	Probiotic, Bactisubtil	n.d.	–	–	+	n.d.	n.d.	lo

*: according to NheB production and toxic activity against CaCo-2 cells (Jessberger et al., 2015). m: medium; hi: high; lo: low; n.d.: not determined.

2.6. Motility assay

Motility of 20 *B. cereus* strains was compared by investigating swimming behavior. In brief, 1 µl overnight culture (OD_{600} = 20) was injected in the center of 53 mm diameter plates containing CGY medium with 0.25% agar. Each strain was tested at least in triplicates. After 24 h incubation at 30 and 37 °C, colony diameters were measured.

2.7. Immunoblotting

A representative subset of six strains - NVH0075–95, F4429/71, F837/76, 7/27/S (potentially/high pathogenic) and RIVM BC 90, MHI 86 (potentially low pathogenic) - was used to test the suitability of selected virulence factors as markers for enteropathogenicity. To this end, mouse monoclonal antibodies against NheA (mAb 1A8), NheB (mAb 1E11), NheC (mAb 3D6) (Dietrich et al., 2005), and sphingomyelinase (SMase; mAb 2A12) (Dietrich et al., 1999), as well as rabbit polyclonal antibodies against collagenase A (ColA) (Abfalter et al., 2016), immune inhibitor metalloprotease (InhA; SE4342, INRA, France) and phosphocholine-specific phospholipase C (PC-PLC; ABIN459259, antibodies-online, Germany) were used.

Samples from filtered culture supernatant were prepared as described above. For Western blots, proteins from the supernatants were precipitated with 100% trichloracetic acid, washed with acetone and resuspended in 30 mM Tris buffer (pH 8.5) containing 7 M urea, 2 M thiourea, 1% serdolit, and 4% CHAPS. The protein concentration was determined using the 2D Quant Kit (GE Healthcare, UK). SDS-PAGE was carried out according to Laemmli (1970) and proteins were subsequently transferred onto a 0.45 µm nitrocellulose blotting membrane (Amersham™ Protran® Premium, GE Healthcare, UK) with blotting buffer (50 mM Tris, 39 mM glycine, 0.0039% SDS), by using a BioRad Transblot SD Semi Dry Transfer Cell (Bio Rad, USA). Calculation of total protein load was carried out by staining with Ponceau S Solution (Applichem, Germany) and was adjusted for each target. 5 µg were loaded for the detection of NheA, NheB, SMase, and PC-PLC, 10 µg for the detection of InhA, and 20 µg for the detection of NheC, respectively.

For Slot blots, a defined volume of supernatant was applied directly. Slot blotting was performed using a Bio-Dot® SF Microfiltration Apparatus (Bio Rad, USA) with a 0.45 µm nitrocellulose blotting membrane (Amersham™ Protran® Premium, GE Healthcare, UK). The volume of applied supernatant was determined by first using 500 µl of the sample with lowest OD_{600} and then by adjusting the other samples via [lowest OD_{600} * 500 µl/OD_{600}], respectively. The protein solutions were loaded into the slots and proteins were transferred to the membrane via vacuum blotting for 45 min. Blocking of unspecific binding was carried out using 5% milk powder solved in 1x TBS at room temperature for 1 h. Primary antibodies were incubated with the membrane at 4 °C overnight and the respective secondary antibody (peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG, Dianova, Germany; Goat Anti rabbit IgG, HRP conjugated, Agrisera, Germany) was incubated with the membrane for 1 h at room temperature. The blots were developed using SuperSignal™ West Pico chemiluminescence substrate (Thermo Scientific, USA). Quantification of blot signals was performed using the Image Quant TL Software (GE Healthcare Life Sciences, UK). Values were calculated as arithmetic mean with standard deviation from two blot experiments.

2.8. Protease activity assay

Casein-specific activity of secreted proteases was determined using the Pierce™ Fluorescent Protease Activity Kit (ThermoFischer, USA), according to manufacturer's instructions, with modifications. The following dilutions of trypsin, based on a 1 µg/ml solution in TBS, were used as standards: 1,000, 750, 500, 250, 100, 75, 50, 25, 10, 0 ng/ml). 75 µl of a FITC casein working solution (10 µg/ml) were added to 75 µl of supernatant sample or standard in a black 96-well plate with transparent bottom (Greiner Bio One, Austria) and incubated for 60 min at 37 °C in the dark. After 10 min of cooling down, 50 µl of assay buffer was added to stabilize the pH value. The fluorescence signal was measured at 485 nm (cut off: 530 nm, 538 nm) in a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices, USA). To determine the metalloprotease activity, 0.15 µl of 0.5 M EDTA (pH 8.0) were added. Final protease activity was depicted as relative fluorescence units (RFU). All values were obtained from two biological samples and two technical replicates, respectively.

2.9. Statistical analyses

Data were statistically validated using the column statistics program of GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). Unpaired *t*-test was applied with two-tailed p values and 95% confidence interval. All data

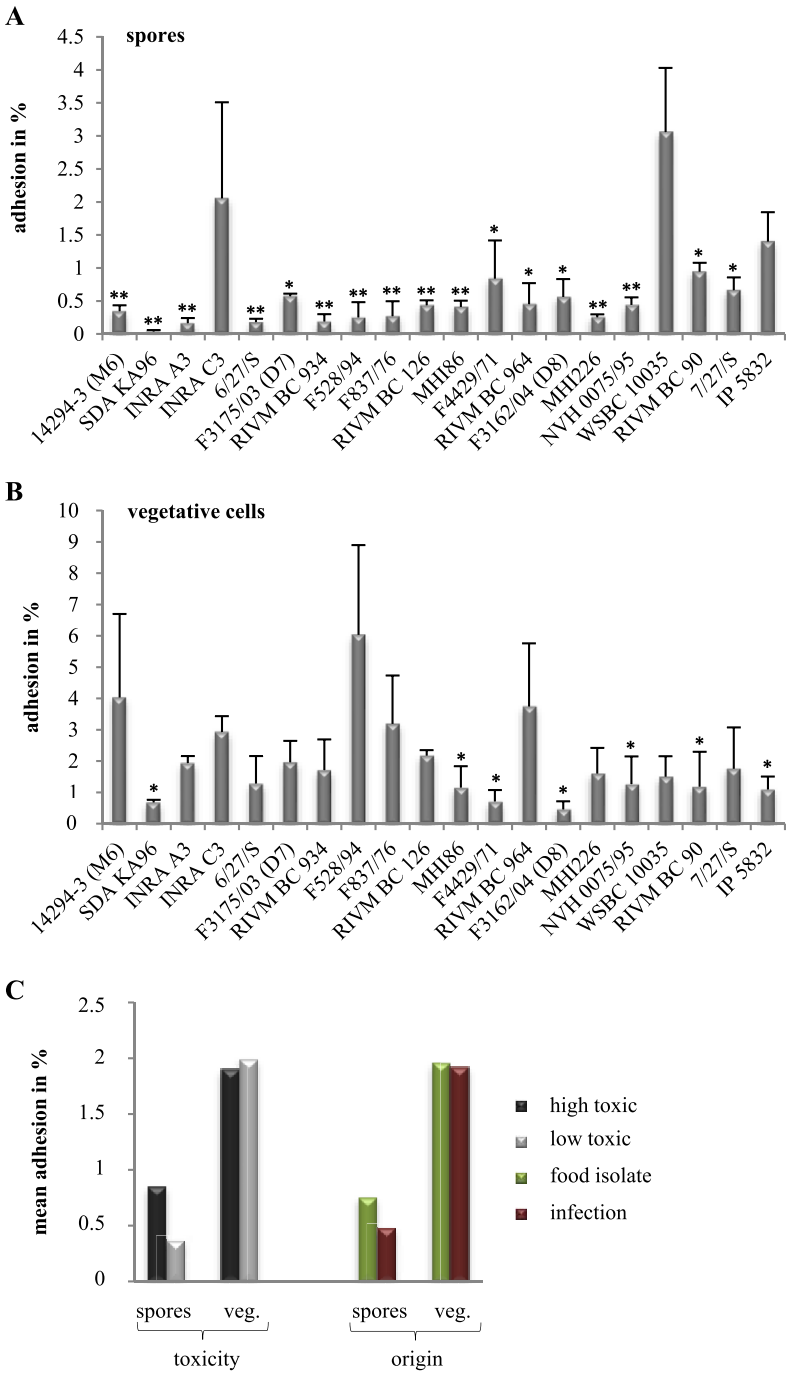

Fig. 1. Adhesion to CaCo-2 cells. Differentiated CaCo-2 cells were infected for 30 min with *B. cereus* spores (MOI 1) or vegetative cells (MOI 10). **A.** Percentage of adhered spores compared for 20 *B. cereus* strains. **B.** Percentage of adhered vegetative cells compared for 20 *B. cereus* strains. Statistically significant differences compared to the respective strain with highest adhesion (spores: WSBC 10035, vegetative cells: F528/94) are shown. *: p value $<$ 0.05, **: p value $<$ 0.01. **C.** Mean adhesion of high and low toxic strains as well as isolates from food samples and from infections.

were compared to the respective "best" strain - with strongest adhesion or germination, highest motility, virulence factor expression or protease activity. Significant differences were marked with * (p value $<$ 0.05), ** (p value $<$ 0.01) or *** (p value $<$ 0.001).

3. Results

3.1. Strain-specific adhesion to CaCo-2 cells

The ability to adhere to human colon epithelial cells was compared for 20 *B. cereus* strains. Ramarao and coworkers showed that *B. cereus* is generally capable of adhesion (Ramarao and Lereclus, 2006). As it is unclear if spore germination occurs in the lumen or at the epithelia, adhesion assays were performed with spores as well as vegetative *B. cereus*. To determine adhesion of spores, CaCo-2 cells were infected with MOI 1 for 30 min. Fig. 1A shows the percentage of adhered spores compared to the inoculum. Generally, spores of all strains were able to adhere, but with clear strain-specific differences. Strains WSBC 10035 and INRA C3, both high toxic strains isolated from pasteurized foods, showed the highest adhesion of 3 and 2%, respectively. With 1.4%,

former probiotic strain IP 5832 followed. Strain SDA KA96 had the lowest adhesion rate with 0.036%. To investigate adhesion of the respective vegetative bacteria, CaCo-2 cells were infected with MOI 10 for 30 min. Preliminary tests revealed no significant differences between MOI 1 and 10 for adhesion of vegetative *B. cereus* (data not shown). Again, all of the tested strains were able to adhere to the CaCo-2 cells (Fig. 1B). F528/94, a low toxic strain, showed the highest adhesion rate with 6%, followed by 14294-3 (M6), RIVM BC 964 and F837/76 (all > 3%). Strains SDA KA96 and F4429/71 adhered comparably low (< 1%). F3162/04 (D8) showed the lowest adhesion rate with 0.45%. Determining the mean of all tested samples, it became obvious that under the chosen conditions vegetative cells generally adhered with higher rates than spores (Fig. 1C). Furthermore, spores of high toxic strains adhered better than spores of low toxic strains and spores of food isolates showed a higher adhesion rate than spores of strains isolated from food infections. No differences could be determined among the vegetative cells.

3.2. Germination

Germination of *B. cereus* spores was tested in CGY, RPMI as well as cRPMI medium, with and without prior heat activation for 10 min at 80 °C. Generally, all tested spores were somehow able to germinate and germination was higher in CGY than in RPMI/cRPMI medium, and higher after previous heat treatment. While germination in CGY full medium varied from 10 to 50%, average germination in RPMI/cRPMI medium plus heat treatment was 2–30%. Only very poor germination was observed in RPMI/cRPMI medium without prior heat treatment. Nevertheless, high strain-specific differences were detected, which is exemplarily shown in Fig. 2. Strains with strong (RIVM BC 934) and weak (probiotic IP 5832) germination in CGY medium were found. In contrast to that, strain MHI86 responded rather to heat treatment than to the chosen medium (Fig. 2A). With approximately 30%, strain 7/27/S showed the strongest germination in RPMI and cRPMI medium, but highly dependent on heat treatment. On the contrary, heat treatment did not accelerate germination of strains F837/76 and RIVM BC 964, which responded better to CaCo-2-treated cRPMI than to RPMI medium (Fig. 2B). Besides newly produced spores, also three year old spore preparations were tested for their germination ability. For example, old spores of strain RIVM BC 934 germinated the same way as freshly prepared ones, while 7/27/S germinated even better in RPMI than in CGY medium, and MHI86 responded less clearly to heat treatment (Fig. 2C). To compare germination rates of all strains, decrease of OD_{620} of all new spores at 20 min (CGY) and 40 min (cRPMI plus heat treatment) was chosen (see Table 2).

3.3. Strain-specific motility

To compare motility of the selected strains, their swimming ability on CGY plates containing 0.25% agar was investigated. While diameters were measured at different time points, the clearest differences were detected after 24 h incubation. At 30 °C, the two strains F3175/03 (D7) and RIVM BC 964, both highly toxic, had reached the plates' edge (maximum diameter 53 mm). On the other hand, strains RIVM BC 934, F528/94 and F837/76 showed no or only poor extension (≤ 2 mm) (Fig. 3A). At 37 °C, swimming diameters of 13 out of 20 strains were increased. Next to F3175/03 (D7) and RIVM BC 964, strains NVH 0075–95, WSBC 10035 (high toxic) as well as 6/27/S and RIVM BC 90 (low toxic) also showed maximum extension (Fig. 3B). Nevertheless, some strains were not affected by the increase of temperature, as for instance 14294-3 (M6) and MHI 226 (medium extension) or RIVM BC 934 and probiotic IP 5832 (no or low extension). Thus, it can be concluded that swimming motility of *B. cereus* is partially temperature-dependent, but highly strain-specific.

3.4. Detailed virulence assessment scheme

To achieve our overall goal, the creation of a holistic virulence assessment scheme for enteropathogenic *B. cereus*, the parameters *spore survival of the stomach passage* (Da Riol et al., 2018), *adhesion of spores* (this study), *germination under simulated intestinal conditions* (this study), *motility* (this study), *adhesion of vegetative cells* (this study), *enterotoxin (NheB) production under laboratory* (Jessberger et al., 2015) *and simulated intestinal conditions* (Jessberger et al., 2017) and *cytotoxicity towards CaCo-2 cells* (Jessberger et al., 2015, 2017) were compared for the set of 20 highly pathogenic and apathogenic strains. To reconcile these different data, the strain with the respective utmost result was set 100%, and the results of the remaining strains were adjusted to that (Table 3). The percentage points of each strain were totaled and according to that, the strain set was reclassified (Table 4I). Strains with a total amount of > 350 percentage points were defined potentially high pathogenic, strains with 240–350 percentage points potentially pathogenic, and strains with < 240 percentage points potentially apathogenic. This classification can serve as the basis for a scientifically substantiated and reliable risk evaluation of enteropathogenic *B. cereus* isolates. It is highly advantageous compared to the former routinely performed risk evaluation mainly based on toxin (NheB) production after cultivation under standard laboratory conditions. The old classification (Table 4. III) for instance overlooks highly toxic strains with mutated *nheB* genes, such as F3162/03 (D8), which occurs in approximately 2% of the isolates (Didier et al., 2015).

3.5. Identifying candidate markers displaying B. cereus cytotoxicity

Investigating all parameters mentioned above is highly time and resource consuming. For routine diagnostics of food samples, an equally reliable, but faster screening scheme is needed. Thus, we investigated *B. cereus* virulence factors as putative candidate markers on a subset of six representative strains (NVH0075–95, F4429/71, F837/76, 7/27/S, RIVM BC 90, MHI 86), according to their correlation with the established toxicity scheme. To this end, the proteins secreted by the bacteria under host mimicking conditions were subjected to Western blotting. The single components A, B, and C of the non-hemolytic enterotoxin (Nhe), sphingomyelinase (SMase), immune inhibitor metalloprotease (InhA), phosphocholine-specific phospholipase C (PC-PLC), and collagenase A (ColA) were tested (Fig. S1). The candidate virulence factors NheA, NheC, InhA, PC-PLC, and ColA showed differences in their expression. However, these differences were rather strain-specific and did not correlate with the enteropathogenic potential of the strains. By contrast, a good correlation to entoropathogenicity was observed for NheB and SMase. Maximum blot intensities were observed after 4 h and 6 h, however, strain F4429/71 showed a decrease after 6 h (Fig. S2). Therefore, NheB and SMase were selected as appropriate markers.

In another preliminary test, the relevance of exoprotease activity for the evaluation of the toxic potential was investigated on this subset of six strains. High toxic strains showed a more rapid and raised activity compared to low toxic strains (data not shown). Maximum activity of the high toxic strains was achieved after 3 h and 4 h, and decreased after 6 h. In contrast, the low toxic strains showed a diminished protease activity after 2 h, 3 h, and 4 h. Activity increased with progressing time, reaching a maximum after 6 h. Hence, exoprotease activity was compared after 4 h of incubation.

3.6. NheB/SMase expression and protease activity of the strain set

To establish diagnostic tools based on NheB/SMase expression and exoprotease activity for discrimination of high and low pathogenic *B. cereus* strains, a panel of the 20 pre-characterized strains was used (see Table 1). All strains were incubated for 4 h under host mimicking conditions and supernatants of strains, collected from the bacteria by centrifugation, were subsequently subjected to immunoblotting and

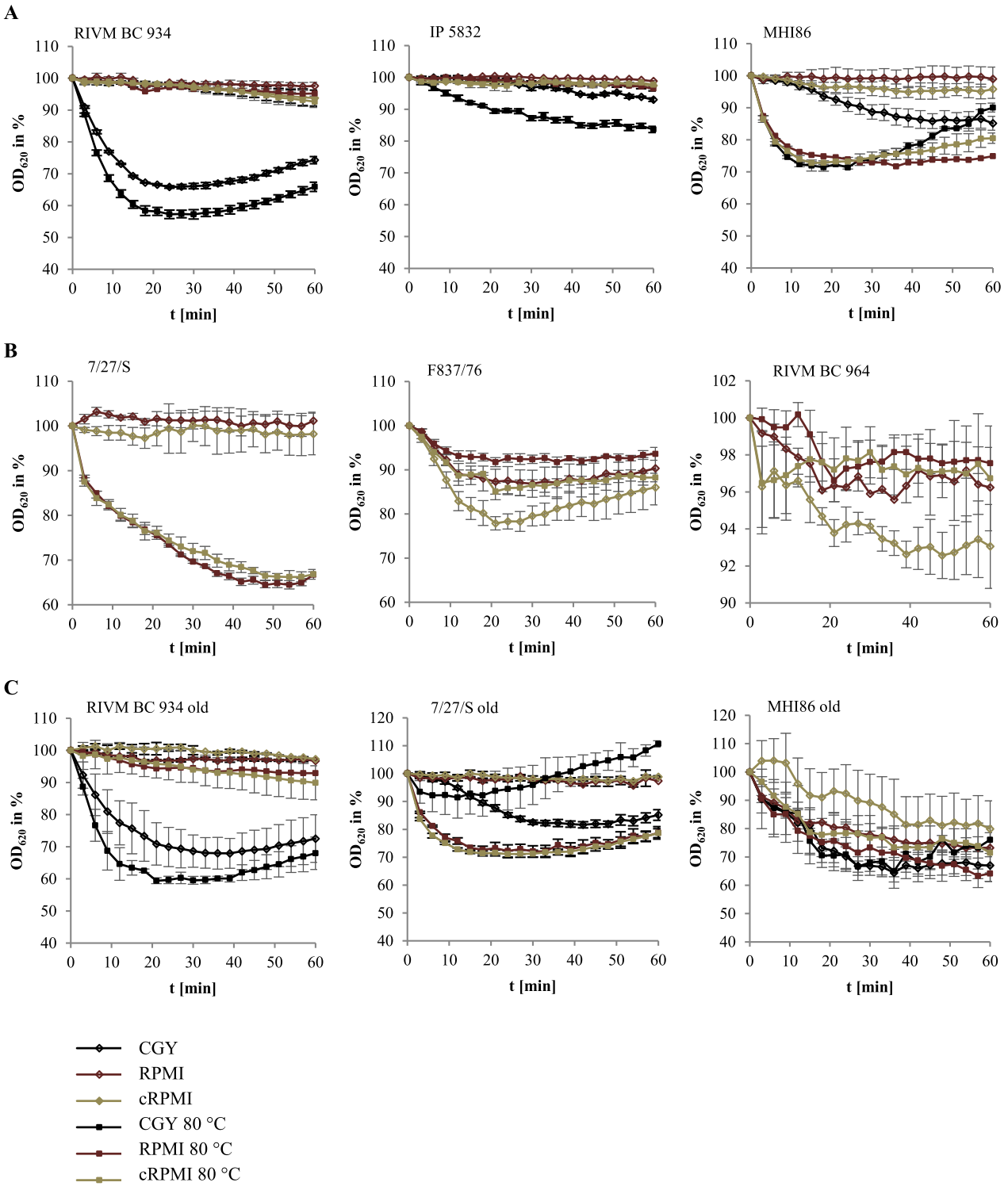

Fig. 2. Germination of *B. cereus* spores. Germination is depicted as the decrease of OD_{620} per unit of time. OD_{620} at time point 0 was set to 100%. One biological replicate (three technical replicates each) is exemplarily shown. **A.** CGY and cRPMI medium. **B.** Comparison of RPMI and cRPMI medium. **C.** Three year old spore preparations.

protease activity tests. Immunoblotting was carried out using a slot blot system with monoclonal antibodies against NheB and SMase. In parallel, exoprotein activity was tested with the commercially available Pierce™ Fluorescent Protease Activity Kit (ThermoFischer, USA), using FITC-labeled casein as a substrate.

Additionally, strains from two food poisoning outbreaks in Austria (2/25, 1/26, 1/27, 1/28, 1/30, 11/6 II AR, 10/12 D; Schmid et al., 2016) with hitherto unknown cytotoxicity and toxin titers were included in order to revise whether their NheB/SMase production and protease activity profile correlates with the epidemiological association

Table 2
Germination of *B. cereus* spores. Germination was measured as the decrease of OD_{620} per unit of time. OD_{620} at time point 0 was set to 100%. To compare germination rates of the 20 tested *B. cereus* strains, OD_{620} (in %) after 20 min incubation in CGY full medium is shown, as well as OD_{620} (in %) after 40 min incubation in cRPMI medium with prior heat treatment. Shown are means and standard deviations of at least two biological replicates, each comprising three technical replicates. p value < 0.05, **: p value < 0.01, ***: p value < 0.001 compared to the strain with best germination (F528/94 for CGY and 7/27/S for cRPMI 80 °C).

B. cereus strain	Germination (OD_{620} in %) 20 min CGY	40 min cRPMI 80 °C
14294-3 (M6)	78.19 ± 0.86***	96.62 ± 2.50***
SDA KA96	78.17 ± 6.8**	93.95 ± 6.53***
INRA A3	92.78 ± 7.11***	85.85 ± 7.17
INRA C3	68.92 ± 1.41***	93.97 ± 3.81***
6/27/S	78.02 ± 8.35**	97.71 ± 0.17***
F3175/03 (D7)	72.03 ± 1.18***	80.28 ± 6.12**
RIVM BC 934	64.26 ± 4.16	94.97 ± 1.62***
F528/94	55.61 ± 2.91	98.00 ± 1.59***
F837/76	80.44 ± 20.44	93.96 ± 4.75**
RIVM BC 126	83.94 ± 4.23***	97.47 ± 0.38***
MHI86	90.80 ± 3.96***	75.84 ± 0.20***
F4429/71	83.66 ± 8.85**	83.44 ± 6.99**
RIVM BC 964	58.25 ± 0.09	98.14 ± 1.70***
F3162/04 (D8)	96.64 ± 2.10***	87.83 ± 3.79***
MHI226	87.54 ± 10.64**	79.23 ± 10.52
NVH 0075/95	94.17 ± 0.90***	76.57 ± 10.53
WSBC 10035	95.89 ± 1.76***	89.09 ± 4.05***
RIVM BC 90	106.4 1 ± 7.94***	95.91 ± 0.79***
7/27/S	91.30 ± 3.62***	67.37 ± 2.22
IP 5832	97.13 ± 2.70***	97.74 ± 1.13***

and, thus, directly with enterotoxicity. The signal intensities of the respective blots are shown in Fig. 4. Most of the strains classified as high pathogenic, such as INRA C3 and RIVM BC 964, showed a strong expression of both proteins. In contrast, the five potentially low pathogenic strains expressed none or only marginal levels of NheB and/or SMase (RIVM BC 934, RIVM BC 90, MHI 86) or produced only one of the toxins in higher amounts (IP 5832: NheB, MHI 226: SMase). Additionally, all potentially low pathogenic strains showed a protease activity below 120 RFU, whereas those of the potentially high pathogenic strains showed values higher than 135 RFU (Fig. 5). The strains classified as potentially toxic showed diverse blot intensities and protease activities. Addition of EDTA inhibited the major part of protease activity which indicates a major contribution of metalloproteases to total proteolytic activity.

3.7. Virulence assessment based on NheB/SMase slot blots and protease activity

In order to establish a virulence assessment based on NheB/SMase expression and protease activity, a point assignment scheme was generated (Table 5). Toxin expression in slot blots (see Fig. 4) was determined as strong (blot signal intensity of 15 or higher), medium (between 5 and 15), low (between 1 and 5), and absent/marginal (below 1). Protease activity (see Fig. 5) was evaluated as strong with an RFU of 300 and above, medium between 200 and 300 RFU, low between 100 and 200, RFU and absent/marginal with an RFU below 100. The rating of the intensities was conducted as follows: 3 = strong, 2 = medium, 1 = low, 0 = absent/marginal. The sum reflects the actual pathogenic potential: 7–9 = potentially high pathogenic, 4–6 = potentially pathogenic, 0–3 = potentially low pathogenic/apathogenic. This classification (Tables 5 and 4. II) is congruent to the detailed virulence assessment scheme (Table 4I) to a large part. However, strains WSBC 10035, 14294-3 (M6) and F3175/03 (D7), classified in Table 4I as potentially high pathogenic, are reduced in rank to potentially pathogenic. Contrary, strain INRA A3 is upgraded from potentially pathogenic to highly pathogenic. Most important, all potentially low pathogenic strains remain within their group. No potentially high pathogenic or pathogenic strain is underestimated following the combinatory scheme of NheB/SMase blot intensities and protease activity. For better comparison, results of the tests systems I, II and III were summarized in Table 4. IV.

Next, we tested this scheme on isolates from foodborne outbreaks in Austria in 2013 (Schmid et al., 2016). Epidemiological association has been previously confirmed for the strains 2/25 (human feces), 1/26 (ragout) and 11/6II AR (soup powder). No epidemiological association could be proven for 1/27 (spinach) and 1/30 (potatoes dumpling). For strains 1/28 (buttermilk) and 10/12 D (parsley), epidemiological association has not been tested. Here, by applying the rating from NheB/SMase blot intensities and protease activity, we were able to confirm the toxic potential of strains 2/25, 1/26 and 11/6II AR, as well as the non-toxicity of strain 1/30. Strain 1/28 was evaluated as potentially high toxic and 10/12 D as potentially toxic. However, strain 1/27 would also be ranked here as potentially high toxic due to strong expression of both NheB and SMase and medium protease activity.

4. Discussion

A reliable risk analysis of products contaminated with *B. cereus* is essential for food industry. On the one hand, food poisoning outbreaks with sometimes severe consequences (Lund et al., 2000; Dierick et al., 2005; Naranjo et al., 2011; Schmid et al., 2016) can be prevented. On the other hand, product charges containing verifiably non-pathogenic *B. cereus* will not be destroyed in vain, which will contribute significantly to food security and prevent economic losses of the manufacturer. In contrast to emetic strains, toxin production in the host intestine has to be considered for enteropathogenic *B. cereus*, which makes risk analyses rather complex. The toxic potential of the strains used in this study has been previously determined by combining NheB toxin titers and cytotoxicity under laboratory (CGY medium, shaking, 30 °C, no CO_2) and intestinal conditions (cRPMI medium, static, 37 °C, 7% CO_2) (Jessberger et al., 2015, 2017). The strain set was complemented by the apathogenic strain IP 5832, which was in use as a probiotic (Kniehl et al., 2003). Simulated intestinal conditions have been previously shown to diminish bacterial growth, but also to trigger toxin production and protein secretion. Also, overall protein secretion was accelerated, with highest protein concentration after 2 h and highest reciprocal titers of NheB after 4 h of incubation in cRPMI medium (Jessberger et al., 2017). Thus, in the current study we maintained these parameters in order to determine the actual pathogenic potential of a strain as accurate as possible. Nevertheless, toxin production and cytotoxicity determined after growth under laboratory conditions (CGY medium) was also kept in the scheme (see Table 3) for reasons of comparison, since the latter conditions are still the method of choice in routine diagnostics.

We showed that a complex virulence assessment scheme including spore survival, adhesion of spores and vegetative cells, germination, motility, toxin production and cytotoxicity towards colon epithelial cells correlates well with a fast and comparably easy system comprising NheB/SMase blots and protease activity. This system could provide the basis for the development of rapid tests for routine diagnostics. So far, there are three systems for enterotoxin detection commercially available: BCET-RPLA kit (Oxoid, UK) detects Hbl L2, TECRA-BDE kit (Tecra International, Australia) detects NheA and Duopath® Cereus Enterotoxins kit detects NheB and Hbl L2. However, these tests provide only a relative indication of the amounts of the specific toxin compounds produced, and do barely allow to estimate the overall enterotoxicity of a given strain (Moravek et al., 2006; Jessberger et al., 2014).

Sphingomyelinases hydrolyze sphingomyelin to ceramide and phosphorylcholine. With acidic, secretory, Mg^{2+}-dependent neutral, Mg^{2+}-independent neutral and alkaline, different types of SMases are known (Goñi and Alonso, 2002). Bacterial SMases can contribute to virulence, as shown for *Staphylococcus aureus*, *Listeria ivanovii* or

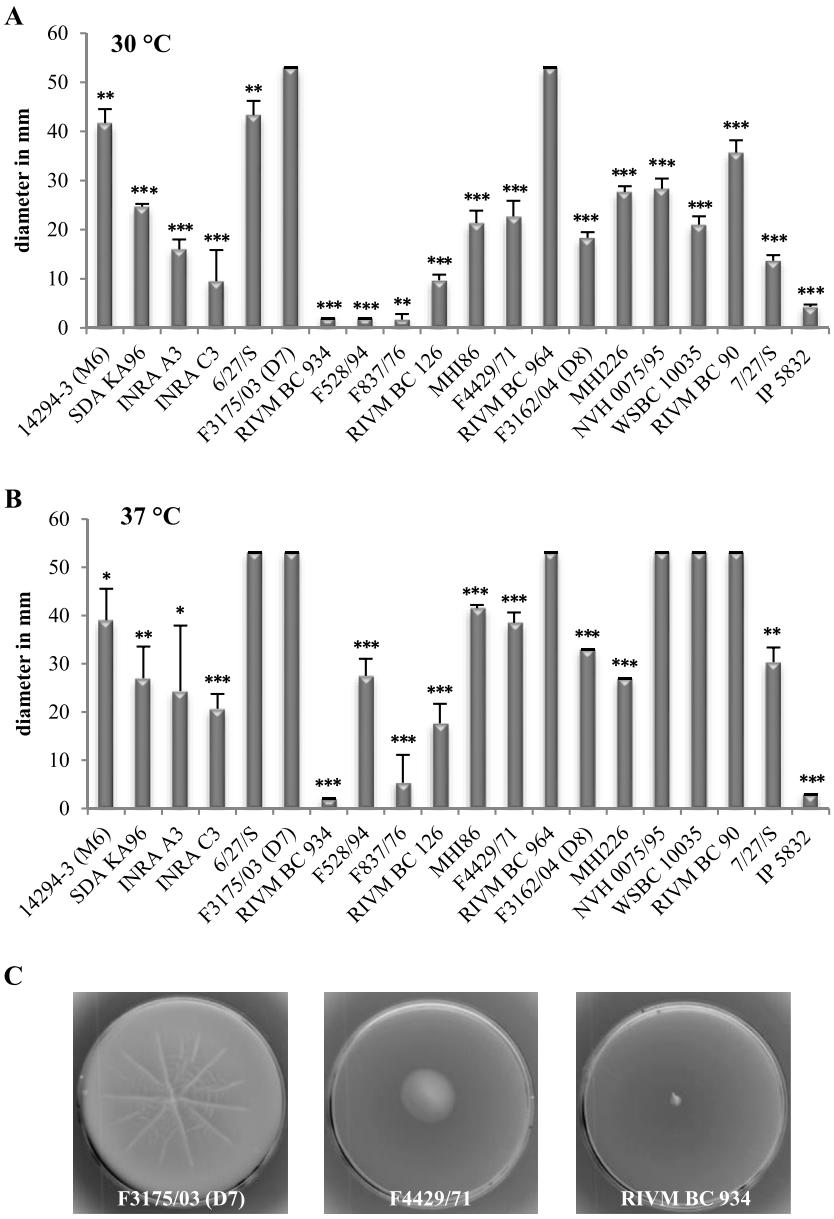

Fig. 3. Motility of the strain set. 1 µl overnight culture (OD_{600} = 20) was injected in the center of CGY plates containing 0.25% agar. Swimming diameters were measured after 24 h incubation. **A.** 30 °C. **B.** 37 °C. Statistically significant differences compared to the respective strain with highest motility (F3175/03 (D7)) are shown. *: p value < 0.05, **: p value < 0.01, ***: p value < 0.001. **C.** Examples of high, medium and low motile strains after 24 h at 30 °C.

Corynebacterium pseudotuberculosis (McNamara et al., 1994; Gonzalez-Zorn et al., 1999; Hayashida et al., 2009; Huseby et al., 2010; Katayama et al., 2013; Flores-Diaz et al., 2016). *B. cereus* SMase is, like the enterotoxin genes, controlled by the global regulator PlcR (Pomerantsev et al., 2003; Gohar et al., 2008) and synergistic interactions between Nhe and SMase have been reported previously. Using the *Galleria mellonella* insect model, it has been shown that SMase enhances Nhe toxicity towards epithelial cells and substantially contributes to mortality of the larvae (Doll et al., 2013). In the present study, SMase was detected in all tested strains, except in the potentially apathogenic RIVM BC 90 and IP 5832. Considering the important role of SMase as virulence factor - in particular, when acting synergistically with Nhe - we reasoned that SMase can constitute a crucial marker protein for virulence assessment, though also requiring the consideration of NheB expression.

NheB is one of the three components of the non-hemolytic enterotoxin (Nhe), which is formed by nearly 100% of the *B. cereus* strains (Anderson Borge et al., 2001; Moravek et al., 2006; Ngamwongsatit et al., 2008). To acquire full cytotoxicity, all three components (A, B and C) are needed. Moreover, a molar ratio of NheA:NheB:NheC of 10:10:1 as well as a concerted binding order of the single components is required (Lund and Granum, 1996; Lindbäck et al., 2004, 2010). Confirming prior studies (Moravek et al., 2006; Jessberger et al., 2014), we observed that NheA and NheC expression did not correlate with cytotoxicity, and differences in blot intensities of these components appeared to be strain-specific only. The expression of NheB (refer to Fig. 4), however, showed rough concordances with strain toxicity, as it was formed by nearly all high and potentially toxic strains and was not detectable in the low pathogenic strains MHI86, MHI226, and RIVM BC 90. The second three-component enterotoxin of *B. cereus*, hemolysin BL (Beecher and MacMillan, 1990; Beecher et al., 1995) also contributes to pathogenicity, but is not suitable as a marker protein as it is produced only by 45–65% of the strains (Senesi and Ghelardi, 2010). The strain set of this study comprised 10 of 20 strains, which produce Hbl. Similarly, CytK2 – a beta-barrel channel forming toxin (Lund et al., 2000) - is produced by strains of different toxicity and therefore not reliable as

Table 3
Summary of the data (in %) of nine assays, in which the 20 pathogenic and apathogenic *B. cereus* strains were applied. To compare the different data, the strain with the respective utmost result (e.g. WSBC 10035 for spore survival) was set 100%, and the results of the remaining strains in each assay were adjusted to that.

B. cereus Strain	Stomach survival[a]	Adhesion spores[b]	Germination[b]	Motility[b]	Adhesion vegetative[b]	NheB (6 h CGY)[c]	NheB (6 h cRPMI)[d]	Toxicity CaCo-2 (6 h CGY)[c]	Toxicity CaCo-2 (6 h cRPMI)[d]	Total
14294-3 (M6)	6.15	11.96	10.35	73.58	60.29	41.54	93.85	50.14	32.13	379.99
SDA KA96	6.98	1.28	18.53	50.94	10.72	66.86	72.52	100.00	46.54	374.37
INRA A3	72.50	5.25	43.37	45.91	32.52	10.35	56.76	19.61	19.29	305.56
INRA C3	32.08	72.04	18.49	38.99	54.34	49.34	100.00	24.80	100.00	490.09
6/27/S	2.29	6.10	7.03	100.00	17.02	11.49	11.51	54.10	32.40	241.94
F3175/03 (D7)	0.31	19.89	60.44	100.00	32.59	27.98	40.05	48.96	25.61	355.83
RIVM BC 934	1.25	6.31	15.42	3.77	25.54	17.11	21.75	44.60	18.99	154.75
F528/94	14.69	7.96	6.12	51.89	100.00	8.08	23.68	38.90	21.98	273.30
F837/76	0.94	7.88	18.51	10.06	45.23	100.00	74.75	68.52	30.00	355.89
RIVM BC 126	0.21	15.26	7.75	33.33	35.60	36.51	43.74	75.67	34.07	282.14
MHI86	0.63	14.14	74.03	78.30	16.55	0.28	0.57	2.89	5.54	192.92
F4429/71	3.65	25.56	50.74	72.64	11.47	28.24	65.52	22.80	21.35	301.97
RIVM BC 964	61.67	15.92	5.69	100.00	59.36	47.92	56.09	53.05	42.63	442.33
F3162/04 (D8)	39.69	18.47	37.30	62.26	5.56	27.29	38.03	42.67	20.27	291.55
MHI226	21.56	8.65	63.66	50.94	25.00	3.49	8.10	31.41	10.84	223.66
NVH 0075/95	4.27	15.29	71.79	100.00	16.41	79.41	83.98	65.95	27.72	464.82
WSBC 10035	100.00	100.00	33.43	100.00	25.22	87.93	41.43	41.00	25.27	554.28
RIVM BC 90	1.04	35.59	12.55	100.00	10.80	3.05	2.49	2.74	1.91	170.16
7/27/S	44.06	22.49	100.00	57.23	22.64	49.63	50.37	45.12	12.50	404.05
IP 5832	10.73	47.40	6.91	5.70	17.30	1.90	6.30	9.60	29.40	135.24

[a] Original data published in Da Riol et al., 2018). Spore survival after 1 h in simulated stomach fluid was used for comparison.
[b] This study.
[c] Original data published in Jessberger et al. (2015).
[d] Original data published in Jessberger et al. (2017).

Table 4
Virulence evaluation of 20 *B. cereus* strains determined with three different test systems. Red: potentially high pathogenic. Orange: potentially pathogenic. Green: potentially apathogenic.

I According to total percentage points achieved in nine different assays (see Table 3)		II According to Slot blots (SMase and NheB) and protease assays		III According to toxin production after cultivation under standard laboratory conditions		IV Summary of test systems I, II and III			
B. cereus strain	Total (%)	*B. cereus* strain	Total points	*B. cereus* strain	NheB Titer[1]	*B. cereus* strain	I	II	III
WSBC 10035	554.28	INRA A3	9	RIVM BC 964	10266	INRA C3			
INRA C3	490.09	INRA C3	8	7/27/S	9011	NVH 0075/95			
NVH 0075/95	464.82	F837/76	8	F837/76	8598	RIVM BC 964			
RIVM BC 964	442.33	SDA KA96	8	RIVM BC 126	7757	7/27/S			
7/27/S	404.05	RIVM BC 964	7	NVH 0075/95	7729	SDA KA96			
14294-3 (M6)	379.99	NVH 0075-95	7	SDA KA96	6481	F837/76			
SDA KA96	374.37	7/27/S	7	WSBC 10035	6205	WSBC 10035			
F837/76	355.89	F3175/03 (D7)	6	F3175/03 (D7)	5157	F3175/03 (D7)			
F3175/03 (D7)	355.83	F3162/03 (D8)	6	F4429/71	4907	F4429/71			
INRA A3	305.56	F4429/71	6	INRA C3	4460	RIVM BC 126			
F4429/71	301.97	14294-3 (M6)	6	14294-3 (M6)	2062	14294-3 (M6)			
F3162/04 (D8)	291.55	WSBC10035	5	6/27/S	1964	INRA A3			
RIVM BC 126	282.14	RIVM BC 126	5	F528/94	1759	F3162/04 (D8)			
F528/94	273.30	6/27/S	4	INRA A3	1299	F528/94			
6/27/S	241.94	F528/94	4	MHI226	930	6/27/S			
MHI226	223.66	RIVM BC 934	3	RIVM BC 934	769	MHI226			
MHI86	192.92	MHI226	3	IP 5832	406	MHI86			
RIVM BC 90	170.16	IP 5832	3	RIVM BC 90	146	RIVM BC 90			
RIVM BC 934	154.75	MHI86	1	MHI86	87	RIVM BC 934			
IP 5832	135.24	RIVM BC 90	1	F3162/04 (D8)	41	IP 5832			

[1]: Titers were determined when a set of 136 *B. cereus* strains was pre-classified. Classification according to NheB titers: high: > 4000; Medium: 2000–4000, low: < 2000 (Jessberger et al., 2015).

a marker.

In this study, we determined that strains classified as high and potentially pathogenic showed an expression of both NheB and SMase, which appears to be stringent for effectual enterotoxicity of *B. cereus*. In particular, both toxins have to be secreted and at least one of the two toxins has to be strongly expressed in order to deploy high toxigenicity. In contrast, strains with low toxic potential showed an expression of none or only one of the toxins or - in case of strain RIVM BC 934 - both

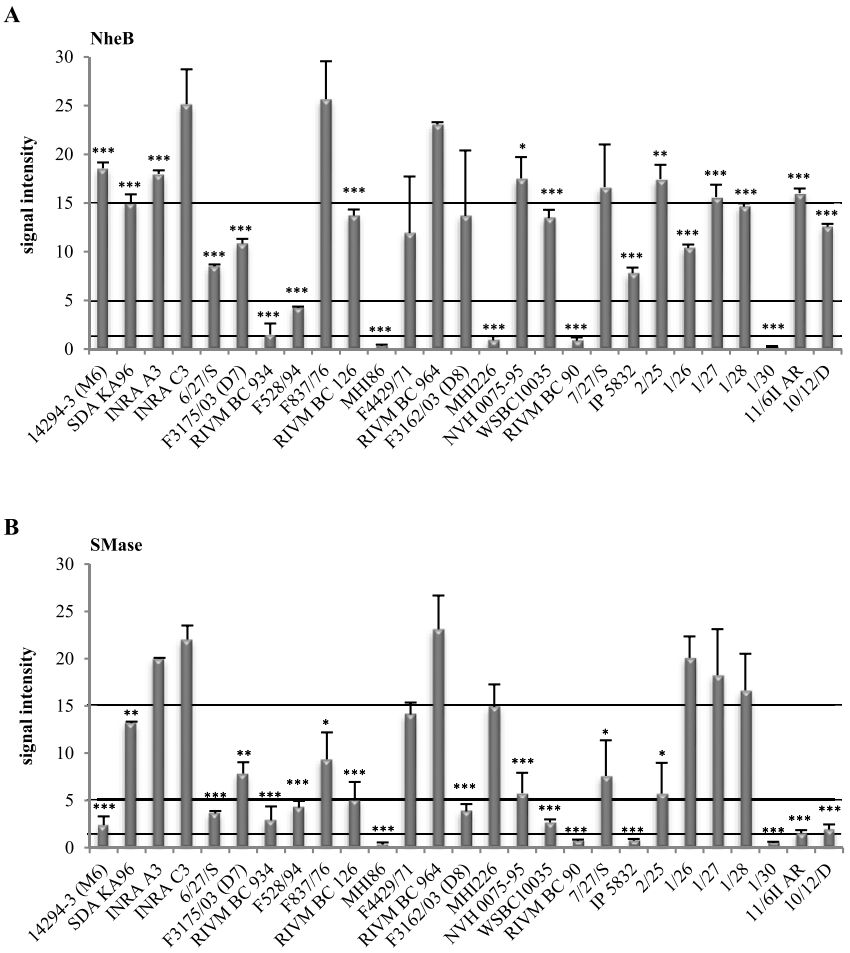

Fig. 4. NheB and SMase expression of all strains tested in this study. A. Quantification of blot signals for NheB using the Image Quant TL Software (GE Healthcare Life Sciences, UK). **B.** Quantification of blot signals for SMase. Statistically significant differences in blot signal intensity are depicted as asterisks and are referred to the strain RIVM BC 964, which showed highest intensity. *: p value < 0.05, **: p value < 0.01, ***: p value < 0.001.

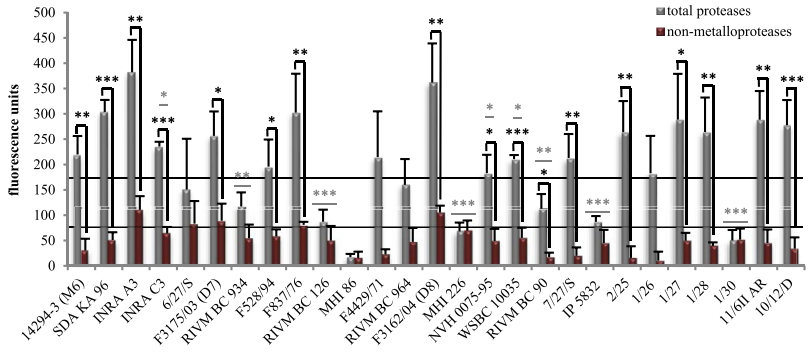

Fig. 5. Protease activity of the *B. cereus* strains tested in this study. Relative fluorescence units are shown. EDTA was added to determine the metalloprotease activity. Expressed results are from two technical replicates, each from two single experiments. Statistically significant differences between total protease and non-metalloprotease activity within each strain are depicted as black asterisks. Significant differences between the total protease activity of each strain, referred to the strain with strongest activity (INRA A3), are shown as grey asterisks with underline. *: p value < 0.05, **: p value < 0.01, ***: p value < 0.001.

with low intensity. Our results fit with the observations of Doll and coworkers (Doll et al., 2013), where pathogenicity of *B. cereus* NheB or SMase single deletion mutants was decreased, and even drastically attenuated when both toxin genes were knocked out.

Moreover, we included exoprotease activity in our virulence assessment scheme. Extracellular proteases play a crucial role for virulence in many pathogens. In *S. aureus*, they are characterized as key mediators of virulence-determinant stability (Kolar et al., 2013). Npr599 and InhA proteases are described as multifunctional pathogenic factors in *Bacillus anthracis* (Chung et al., 2006). CwpFM (EntFM) peptidase plays an important role in motility, adhesion and virulence of *B. cereus* (Tran et al., 2010). The InhA metalloproteases have also been described as important pathogenicity factors of *B. cereus*, being involved in macrophage escape, toxicity and virulence (Guillemet et al., 2010). The results of protease activity assays in the present work generally complement the pattern observed by NheB and SMase intensities. The exception proves the rule, e.g. as shown for strain RIVM BC 964, displaying a low proteolytic activity. However, NheB and SMase expression were distinctly strong, thus rendering this strain as still highly toxic. On the other hand, proteolytic activity of each of the potentially apathogenic strains was diminished compared to those of all other strains.

By following this virulence assessment combined of NheB/SMase blots and exoprotease activity, we could observe a shift of certain

Table 5
Summary of slot blot analyses (NheB and SMase) and protease assays. 20 pre-characterized *B. cereus* strains were investigated (Jessberger et al., 2015, 2017), as well as seven isolates from a food poisoning outbreak in Austria (Schmid et al., 2016). Results of each test were classified as strong (= 3 points), medium (= 2 points), weak (= 1 point) or 0 and totaled. 7–9 points (red): potentially high pathogenic. 4–6 points (orange): potentially pathogenic. 0–3 points (green): potentially apathogenic.

B. cereus strain	Nhe B[a]	SMase[a]	Protease activity[b]	Score
INRA A3	3 (18)	3 (20)	3 (382)	9
INRA C3	3 (25)	3 (22)	2 (235)	8
F837/76	3 (25)	2 (9)	3 (302)	8
SDA KA96	3 (15)	2 (13)	3 (304)	8
RIVM BC 964	3 (23)	3 (23)	1 (160)	7
NVH 0075-95	3 (18)	2 (6)	2 (183)	7
7/27/S	3 (17)	2 (8)	2 (213)	7
F3175/03 (D7)	2 (10)	2 (8)	2 (256)	6
F3162/03 (D8)	2 (14)	1 (4)	3 (362)	6
F4429/71	2 (12)	2 (14)	2 (215)	6
14294-3 (M6)	3 (19)	1 (2)	2 (220)	6
WSBC10035	2 (14)	1 (3)	2 (210)	5
RIVM BC 126	2 (13)	2 (5)	1 (87)	5
6/27/S	2 (9)	1 (4)	1 (150)	4
F528/94	1 (4)	1 (4)	2 (195)	4
RIVM BC 934	1 (2)	1 (3)	1 (118)	3
MHI226	0 (0.9)	3 (15)	0 (68)	3
IP 5832	2 (8)	0 (0)	1 (87)	3
MHI86	0 (0)	1 (3)	0 (17)	1
RIVM BC 90	0 (0)	0 (0)	1 (113)	1
1/27	3 (15)	3 (18)	2 (288)	8
2/25	3 (17)	2 (6)	2 (264)	7
1/26	2 (10)	3 (20)	2 (182)	7
1/28	2 (14)	3 (17)	2 (264)	7
11/6II AR	3 (16)	1 (1)	2 (288)	6
10/12/D	2 (13)	1 (2)	2 (288)	5
1/30	0 (0)	0 (0)	0 (50)	0

[a] Slot blot signal intensities for NheB and SMase > 15: 3 points, 5–15: 2 points, 1–5: 1 point, < 1: 0 points.
[b] Relative fluorescence units (protease activity) > 300: 3 points, 200–300: 2 points, 100–200: 1 point, < 100: 0 points.

strains, from formerly classified as potentially toxic to highly toxic (INRA A3) and vice versa (WSBC 10035, 14294-3 (M6), F3175/03 (D7), when compared to the virulence assessment comprising also phenotypic attributes (Table 4). However, no shift was observable in potentially low or apathogenic strains. Most important, no toxic strain could be rendered as false negatively apathogenic, which might be fatal in terms of food safety. We could also verify this virulence assessment scheme by using seven additional strains from two food outbreaks in Austria with previously unknown toxicity (Schmid et al., 2016). With the exception of one strain, three strains (2/25, 1/26, 11/6II AR) with epidemiological association would be ranked as highly or potentially toxic. One strain (1/30) with no epidemiological association showed no NheB/SMase expression or protease activity as well. Two strains with undetermined epidemiological association could be assigned as highly (1/28) and potentially toxic. This outcome depicts that epidemiological association does not necessarily give information on the toxicity of a strain. Hence, in order to ensure confident predictions, the pathogenic potential has to be determined as proposed in the current study.

In conclusion, this study provides the first holistic virulence assessment scheme for enteropathogenic *B. cereus*, including the most important steps of a foodborne infection - survival of the stomach passage, adhesion to epithelial cells, germination, motility, toxin production and cytotoxicity. An analogically precise and credible classification of the tested strains was achieved by determining NheB and SMase expression and exoprotease activity. These assays provide the fundament for rapid and easy to use tests in routine diagnostics of enteropathogenic *B. cereus*. To this date, the combinatory approach presented in this study is the most accurate and rapid test system for determining the toxic potential of food-borne enteropathogenic *B. cereus*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103276.

Author contributions statement

NJ and CDR were responsible for spore preparation, motility and adhesion experiments, and virulence assessment. MK and TB performed protease and slot blot assays including virulence assessment. VS and TB also prepared spores and performed germination assays. NJ and MK wrote the manuscript. EM, RD and MES were involved in study design and writing of the manuscript.

Conflicts of interest

The authors declare that the research was conducted in the absence of any conflict of interest.

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