Concerted Action of Sphingomyelinase and Non-Hemolytic Enterotoxin in Pathogenic Bacillus cereus

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Abstract

Bacillus cereus causes food poisoning and serious non-gastrointestinal-tract infections. Non-hemolytic enterotoxin (Nhe), which is present in most B. cereus strains, is considered to be one of the main virulence factors. However, a B. cereus ΔnheBC mutant strain lacking Nhe is still cytotoxic to intestinal epithelial cells. In a screen for additional cytotoxic factors using an in vitro model for polarized colon epithelial cells we identified B. cereus sphingomyelinase (SMase) as a strong inducer of epithelial cell death. Using single and double deletion mutants of nhe, the gene encoding for SMase, and nheBC in B. cereus we demonstrated that SMase is an important factor for B. cereus cytotoxicity in vitro and pathogenicity in vivo. SMase substantially complemented Nhe induced cytotoxicity in vitro. In addition, SMase but not Nhe contributed significantly to the mortality rate of larvae in vivo in the insect model Galleria mellonella. Our study suggests that the role of B. cereus SMase as a secreted virulence factor for in vivo pathogenesis has been underestimated and that Nhe and SMase complement each other significantly to cause full B. cereus virulence hence disease formation.

Introduction

Bacillus cereus is a spore forming Gram-positive bacterium that is found in natural habitats like soil and plants. Their spores can easily enter the food processing chain due to heat and dryness resistance. B. cereus is commonly accepted as a food borne pathogen causing mostly mild food borne gastroenteritis. Nevertheless, fatal outbreaks of B. cereus food poisoning [1,2] and local and systemic non-gastrointestinal-tract infections (e.g. endophthalmitis, pneumonia, sepsis) in humans have been reported [3]. Two types of gastrointestinal diseases can be distinguished. Ingestion of B. cereus strains results in mild food borne gastroenteritis, usually associated with virulence factors like hemolysin II and the immune inhibitor A family have been associated with virulence in vivo in the insect host [13,14], whereas major B. cereus enterotoxins such as Nhe, Hbl and CytK were poorly examined.

Expression of the secreted enterotoxins Nhe, Hbl and CytK is controlled by the PlcR regulon via a quorum sensing system [15]. Deletion of plcR abolishes in vitro cytotoxicity and reduces significantly in vivo pathogenicity in mice and insects [12]. PlcR regulates more than 40 genes including degradative enzymes, proteases and phospholipases such as phosphatidylinositol-specific phospholipase C (PI-PLC), phosphatidylcholine-specific phospholipase C (PC-PLC) and sphingomyelinase (SMase) [15]. Synergistic interaction of PC-PLC and SMase for complete lysis of human erythrocytes has been demonstrated and therefore, both enzymes have been proposed to form a cytolytic unit, named cereolysin AB [16]. B. cereus sphingomyelinase is structurally related to Staphylococcus aureus beta toxin, Clostridium perfringens alpha toxin and the sphingomyelinase of the intracellular pathogen Listeria ivanovii [16,17]. Sphingomyelinase is a metal ion-dependent phospholipase that hydrolyzes sphingomyelin in the plasma membrane to phosphocholine and ceramide. Recently gangliosides, especially NeuAcα2-3Galβ1-4Glcβ1-1ceramide (GM3), have been shown as a direct binding site for SMase. The sialic acid of GM3 serves as a cell receptor for binding the β-hairpin region of SMase [18].
Oda et al. demonstrated that B. cereus SMase is an important virulence factor for septicemia. B. cereus strains displaying SMase activity are able to grow in vivo in mice after intraperitoneal injection in contrast to strains without SMase activity [19]. Earlier work by Beecher et al. suggested that SMase acts in connection with the hemolytic enterotoxin complex Hbl [20]. However, hbl genes are restricted to certain strains whereas the nhe operon encoding the so called non-hemolytic enterotoxin complex Nhe has been found in all strains tested so far [21–23]. Nhe is a pore-forming toxin that requires the expression of its individual enterotoxin components Nhe A, B and C in a certain ratio (10:10:1) [24] and specific binding order for full in vitro cytotoxicity [25]. At least two or three Nhe components are necessary for the formation of functional membrane pores depending on the target cell type [26]. After binding of NheC and NheB to the cell surface a conformational change might occur that allows subsequent binding of NheA, resulting in cell lysis [25]. It is assumed that Nhe is the major enterotoxin playing a key role in B. cereus induced diarrhea [27] but a synergistic interaction between SMase and Nhe in B. cereus virulence has not been studied so far. Therefore, we examined the interaction of Nhe and SMase for in vitro cytotoxicity and in vivo pathogenicity. In this work, we provide evidence that B. cereus SMase enhances Nhe cytotoxicity in an in vitro model for polarized colon epithelial cells. SMase in vitro cytotoxicity depends on the presence of Nhe. Furthermore, in the insect model Galleria mellonella deletion of the SMase gene sph but not nheBC significantly reduced larvae mortality. Inactivation of both gene loci indicated that SMase and Nhe cooperate in B. cereus pathogenicity in vivo.

**Materials and Methods**

**Bacterial Strains, Plasmids and Culture Conditions**

*Bacillus cereus* NVH 0075-95 [28], its isogenic mutant strains and *E. coli* strain were routinely grown in Luria-Bertani (LB) broth or on LB agar plates at 30°C or 37°C, respectively. For cytotoxicity screening bacterial supernatants were collected from 100 ml cultures in LB broth (37°C, 150 rpm) 8 h after inoculation with 10^5 colony-forming units (CFU/ml) from a 16-hour pre-culture (corresponding to early stationary phase). When required, bacterial supernatants were concentrated up to 30-fold at 4°C using Amicon® centrifugal filter devices (10 kDa, Millipore). Supernatants were stored at –80°C. Unless stated otherwise, cultures were grown in 200 ml flasks and optical density at 600 nm (OD_{600}) was recorded. Samples exceeding an OD_{600} of 1 were diluted 1:10 and extrapolated.

Where appropriate a single or a combination of the following antibiotics was added to the media: ampicillin (100 µg/ml), kanamycin (50 µg/ml), spectinomycin (100 µg/ml), polymyxin B (50 µg/ml), erythromycin (3 µg/ml), chloramphenicol (5 µg/ml) or tetracycline (10 µg/ml). All bacterial strains and plasmids constructed and used in this study are listed in supplemental Table S1 and S2.

For phenotypic characterization of *B. cereus* wild type (WT) and mutant strains MYP agar (mammitol egg yolk polymyxin; Oxoid) and Columbia agar (containing 5% sheep blood, Oxoid) was used. Therefore, small amounts of colony material were transferred to the respective agar plate surface using the tip of a needle and incubated at 30°C for 24 h. To test for extracellular cross-complementation of hemolytic activity, *B. cereus* NVH 0075-95 WT, ΔnheBC, Δsph and ΔnheBCΔsph mutants were streaked out crosswise on sheep blood agar plates as described before [16] similar to diagnostic CAMP test and incubated at 30°C for 24 h.

**General Molecular Methods**

For cloning, genomic DNA of *B. cereus* NVH 0075-95 served as a template. DNA isolation, manipulation and transformation in *E. coli* was carried out in accordance with standard protocols [29]. All oligonucleotides used in this study are listed in Table S2. A high-fidelity Platinum® Taq DNA polymerase (Invitrogen) was used for DNA amplification and DNA manipulations were verified by sequencing.

**Epithelial Cell Cytotoxicity**

*Ptk6* null epithelial cells (Ptk6) from mouse colonic mucosa were cultured as outlined previously [30]. In cytotoxicity assays, 1×10^6 Ptk6 cells were seeded onto 6-well plates and grown until confluence. For in vitro cytotoxicity tests Ptk6 cell monolayers were washed twice with DPBS and treated with either vegetative cells of various *B. cereus* strains (Table S3) at an MOI of 1 (2×10^9 bacteria) or sterile bacterial supernatant at 37°C/5% CO₂ atmosphere. IEC Changes in intestinal epithelial cell (IEC) morphology, cell rounding and % cell detachment was monitored over time using light microscopy.

**Identification of Cytotoxic Proteins Secreted by B. cereus**

Concentrated bacterial supernatant of *B. cereus* NVH 0075-95 ΔnheBC was dialyzed against PBS (pH 7.4) and total protein concentration was determined using a BCA protein assay (Thermo Scientific Pierce). 1.64 mg of secreted proteins was fractionated by gel filtration using a Superdex-75 10/300 GL column in a AKTA Purifier system (GE) with a flow rate of 0.2 ml/min. Proteins were detected by their absorbance at 280 nm. Fractions were tested for cytotoxic activity on Ptk6 cell monolayers. After SDS-gel separation, silver stained protein bands were analyzed by LC/MS/MS on a ThermoFisher LTQ Orbitrap XL mass spectrometer by NextGen Sciences, Ann Arbor, USA. Protein identifications were accepted according to NextGen Science's guidelines (greater than 90.0% probability, at least 2 identified peptides). Protein probabilities were assigned by the Protein Prophet algorithm [31].

**Construction of B. cereus NVH 0075-95 sph Deletion Mutants**

*Sphingomyelinase* null mutants and complemented strains were generated as described before [32]. In brief, DNA regions of approximately 1200 bp flanking the *sph* gene were amplified by PCR using primers shown in Table S2, primarily inserted into the cloning vector pCR 2.1 TOPO flanking a chloramphenicol resistance cassette. The construct was cut with *SacI/HindIII* and inserted in the similarly digested conjugative suicide vector pAT113. The resulting vector pAT113Asph/cm was transformed into *B. cereus* NVH 0075-95 WT and ΔnheBC via conjugation using the donor strain *E. coli* JM83/pRK24 as described elsewhere [33]. Transconjugants were screened for chloramphenicol resistance and erythromycin sensitivity. To confirm gene deletion and integration of the resistance cassette, immunoblotting, PCR and sequencing using the primer pair sph_Sac_F/sph_Hind_R, Cm_F/Cm_R_Not or InCm2_F/sph_downstream_R were carried out. The *sph* deletion mutants were designated *B. cereus* NVH 0075-95 Δsph and ΔnheBCΔsph.

**Complementation of sph Deletion Mutants**

To complement *sph* deletion mutants, plasmids containing one of the two putative *ple-sph* operon promoter regions *Ppbc* and *Ptph* directly upstream of the *sph* coding sequence were constructed. Using the primer pair *Ppbc_for/Ppbc_rev* a 538 bp region
upstream of the plc gene (promoter Ppplc-sph of the plc-sph operon) was amplified from B. cereus NVH 0075-95 genomic DNA. The PCR product was digested with EcoRI and Sall and was ligated in the similarly digested pAD123 vector containing an additional tetracycline resistance cassette (pAD/tet). In a second PCR reaction the promoterless sph coding sequence was amplified using the primer pair sph_Sac_F/sph_Hind_R. After amplification the PCR product was cloned into the SaeI/HindIII restriction site of the same plasmid leading to the shuttle-vector pAD/sph/Ppplc/tet. As control the sph gene including a 499 bp upstream region (putative promoter Ppplc described before [34,35]) was PCR amplified using the primer pair Ppplc1_for/sph_Hind_R. After restriction digest the PCR product was inserted into the EcoRI-HindIII site of pAD/tet giving rise to a plasmid designated pAD/sph/Ppplc/tet. Both resulting plasmids were propagated in non-methylyating E. coli INV110 and introduced into the B. cereus NVH 0075-95 sph single and triple mutant strains by electroporation as described previously [36]. PCR analysis with Tet’-specific primers confirmed the introduction of the plasmids into the generated mutant strains B. cereus NVH 0075-95 Δsph comPpplc, Δsph comPPpplc, ΔsphΔBCΔsph comPPpplc and ΔnheBCΔsph comPPpplc. Expression of SMase was confirmed by immunoblotting and SMase activity assay.

Flow cytometric Analysis of B. cereus Cytotoxicity

For flow cytometric analysis, 1 × 10⁶ Ptk6 cells were seeded onto 6-well plates and grown until confluence. For the assessment of bacterial cytotoxicity, Ptk6 cell monolayers were washed twice with DPBS, treated with serial dilutions of bacterial supernatant in RPMI 1640. After 4 h at 37°C/5% CO₂, epithelial cells were washed, trypsinized and stained with Propidium iodide (Biozol) to detect necrotic cells. Samples containing 1–2 × 10⁶ cells were analyzed for total and dead cell numbers using a Gallios flow cytometer (Beckman Coulter). To decipher the effect of SMase for B. cereus virulence in vitro, confluent Ptk6 cells were treated with sublethal dilutions (1:16) of supernatant of B. cereus NVH 0075-95 Δsph or ΔnheBCΔsph supplemented with 0, 0.05, 0.1 and 0.2 U/ml of recombinant SMase (Sigma, S9396). To neutralize Nhe activity in the supernatant of Δsph, a monoclonal anti-NheB antibody (1E11) kindly provided by Richard Dietrich [27] was added to wells (10 µg/well) supplemented with 0, 0.1 and 0.2 U/ml of recombinant SMase. After 4 h incubation at 37°C/5% CO₂, cells were analyzed by flow cytometry as described above.

PC-PLC and SMase Activity Assay

For enzyme activity assays supernatants of B. cereus wild type, the nheBC and sph mutants and their complemented derivatives cultivated in LB broth at 37°C were harvested (6500 g, 4 min, 4°C) at an OD₆₀₀ of 4 and 7 corresponding to transition and early stationary growth phases, respectively. Using an Amplex® Red Sphingomyelinase Assay Kit (Invitrogen/Molecular Probes) production of sphingomyelinase was determined, while synthesis of phosphatidylcholine-specific phospholipase C was quantified with the EnzChek Direct Phospholipase C Assay Kit (Invitrogen/Molecular Probes) following the manufacturer’s protocol. PC-PLC activity assay was carried out as described before [37] and sphingomyelinase assay was done in accordance except for the following changes. For SMase activity the reactions were developed for 60 min at 37°C in a light-protected microplate thermo-shaker and fluorescence was measured with a Wallac 1420 Victor² multilabel plate reader (Perkin Elmer) at excitation/ emission wavelengths of 530/585 nm. Samples of three independent cultures were examined for their phospholipolytic activities. To account for different protein concentrations of the samples enzyme activity was normalized to protein concentration of the respective supernatant using a Bradford-based protein assay (Roti-Quant, Carl Roth GmbH), resulting in mU per µg of protein.

Immunoblotting of SMase

Protein samples were prepared from bacterial supernatants of B. cereus NVH 0075-95 WT, isogenic mutant and complemented strains. 4 µg of total B. cereus protein were separated via 10% SDS/PAGE as described before [38]. After transfer to a PVDF membrane (Roche) B. cereus SMase was detected using a rabbit polyclonal anti-BcSMase antibody (1:1000) kindly provided by Jun Sakurai [39]. An Alkaline-Phosphatase-conjugated goat-anti-rabbit IgG (1:30000) (dianova) was used as second antibody for chromogenic SMase detection.

Insect Injection Experiments

Galleria mellonella (G. mellonella) larvae were purchased from Kerf (Unna, Germany) and kept at 15°C. Thirty last-instar G. mellonella larvae per condition, weighing 200–400 mg were kept in groups of 10 larvae per box at 15°C. Injection of 5 µl suspensions of vegetative bacteria was done intrahemocoeically into the base of the last left proleg of the larvae. 1.2–2.2 × 10⁷ CFU per larva for B. cereus NVH 0075-95 WT, ΔnheBC, Δsph, ΔnheBCΔsph and the complementation mutant ΔnheBCΔsph comPPpplc and 7.0 × 10⁶ CFU per larva for E. coli DH10B were injected, respectively. Bacterial cell counts of the injected suspension were determined for all strains by plating serial dilutions of the inocula on appropriate medium. G. mellonella survival was determined daily over 7 days at 15°C. Larvae were considered dead if they failed to respond to stimulation with a forceps. All tests were run in duplicates with 30 larvae per experiment and condition and they gave similar results for each experiment.

To determine the time-course of bacterial multiplication within G. mellonella larvae, 5 larvae of each condition (different injected bacterial strains) were homogenized individually at time-points indicated in the diagram and serial dilutions of the homogenate were plated on LB agar plates for determination of CFU per larva. Larvae were surface-cleaned with 70% Ethanol prior to homogenization. Bacterial growth monitoring was done in two independent experiments.

Software and Statistical Analysis

Mean values and S.E. were calculated from at least three independent experiments. For statistical analysis, 2-tailed Student’s-t-test was used, where applicable, to determine statistically significant differences as indicated in figure legends (GraphPad Prism, Graph Pad Software). Figures were assembled with Photoshop CS and Illustrator CS (Adobe Systems). Raw data of the insect experiments were analyzed using GraphPad Prism (version 5.0) software and plotted according to Kaplan-Meier. Curves were compared for statistical differences using log-rank analysis, which generates a P value testing the null hypothesis that the survival curves are identical. P values of 0.05 or less were considered significantly different from the null hypothesis.

Results

PlC-mediated Cell Cytotoxicity in Intestinal Epithelial Cells

In order to analyze the role of cytotoxicity factors in B. cereus infection, 14 different B. cereus strains, which vary in their known toxin gene profile (Tab. S1), were tested for cell cytotoxicity in polarized intestinal epithelial cells (IECs) in vitro. As a cell culture
model for polarized IEC we used colon epithelial cells derived from Ptk6 null mice [30].

Infection of Ptk6 IEC with various B. cereus strains at an MOI of 1 caused rapid cell death with a complete detachment of cells from the tissue culture plate within 4 h (Fig. 1). Although the beginning of cellular rounding and detachment varied between 2 and 4 h there was not a gross difference between strains tested. Even two B. cereus strains isolated from two different probiotic formulas used in humans and piglet feeding [40–43], B. cereus var. toyoi and IP5832, revealed similar cytotoxicity compared to B. cereus NVH 0075-95 isolated from a food borne outbreak associated with high patient mortality [1] or the nhe reference strain B. cereus NVH 0075-95 (Fig. 1). Cellular cytotoxicity was mediated by a secreted B. cereus factor as rapid rounding and detachment in IEC was induced by bacterial supernatants alone. (Fig. S1). Infection of two different human colonic epithelial cell lines Caco-2 and T84 showed similar results (data not shown).

Common to all B. cereus strains tested is the presence of the nhe operon genes (Tab. S1). A B. cereus NVH 0075-95 nheBC mutant (ΔnheBC; kindly provided by Simon P. Hardy [44]) as well as its supernatant caused a delay of onset of cellular rounding by 1 h compared to wild type, but IEC still detached completely after 4 h and 6 h, respectively (Fig. 1 and S1). Due to nheB truncation and nheC deletion B. cereus NVH 0075-95 ΔnheBC is unable to produce a functional Nhe toxin, since maximal cytotoxic activity of Nhe depends on at least two Nhe components [24]. Cytotoxicity was completely abolished using a B. cereus mutant strain lacking the PlcR regulon [B. cereus F4872/10 ΔplcR] [32]. These data indicate that additional PlcR regulated proteins contribute to IEC cytotoxicity. As B. cereus NVH 0075-95 does not express enterotoxins like Hbl or CytK, we screened for other cytotoxic components in the B. cereus NVH 0075-95 ΔnheBC supernatant using fast protein liquid chromatography (FPLC). Proteins contained in the bacterial supernatant were separated according to their size on a Superdex-75 10/300 GL gel filtration column (Fig. 2A). Fractions 11–18 contained most of the extracellular bacterial proteins. IEC cytotoxicity was confined to three fractions by infecting polarized epithelial monolayers with each of the fractions of the bacterial supernatant of B. cereus NVH 0075-95 ΔnheBC. Only fractions 12–14 conveyed complete epithelial cell detachment. SDS gel electrophoresis analysis of fractions 8–14, as well as unfractionated supernatant is shown in Fig. 2B and 2C. Comparison of proteins contained in the cytotoxic fractions 12–14 with unfractionated mutant and wild type B. cereus supernatants revealed a 34 kDa and a 25 kDa protein band (indicated in Fig. 2B by red asterisk) that were absent in the non-cytotoxic supernatant of B. cereus F4810/72 ΔplcR mutant (Fig. 2C). Protein identification of the two silver stained protein bands was carried out by NexGen Sciences (AnnArbor, USA) using LC/MS/MS on a ThermoFisher LTQ Orbitrap XL mass spectrometer. Mass spectrometry analysis of the 34 kDa protein identified sphingomyelin phosphodiesterase (sphingomyelinase) of B. cereus (NCBI accession number NP_002336808) by two unique peptide sequences, (K)DHANPSF-VENK(V) and (K)VQYVFANGCGPDNLSNK(G), and a sequence coverage of 74%. The 25 kDa protein band was identified as hybrid cereolysin AB (NCBI accession number CAA45501).

### Table 1: B. cereus strains and cytotoxic traits

| Time [h] | F4810/72 | F4810/72 ΔplcR | NVH 0075-95 | NVH 0075-95 ΔnheBC | NVH 0230-00 | NVH 0861-00 | NVH 0291-06 | F3837/65 | F3605/73 | F4437/73 | INRA/21 | RVW BC67 | WSEBC 10030 | WSEBC 10030 var. toyoi | IP 5832 |
|----------|----------|----------------|--------------|-------------------|-------------|-------------|-------------|----------|----------|----------|---------|-----------|---------------|---------------|-------------|-----------|
| 0.5      |          |                |              |                   |             |             |             |          |          |          |         |           |                |               |             |           |
| 1.0      |          |                |              |                   |             |             |             |          |          |          |         |           |                |               |             |           |
| 1.5      |          |                |              |                   |             |             |             |          |          |          |         |           |                |               |             |           |
| 2.0      |          |                |              |                   |             |             |             |          |          |          |         |           |                |               |             |           |
| 2.5      |          |                |              |                   |             |             |             |          |          |          |         |           |                |               |             |           |
| 3.0      |          |                |              |                   |             |             |             |          |          |          |         |           |                |               |             |           |
| 4.0      |          |                |              |                   |             |             |             |          |          |          |         |           |                |               |             |           |

**Figure 1.** Cytotoxic effects of various B. cereus strains on intestinal epithelial cells (IEC). Ptk6 cells were treated with 14 different B. cereus strains and two isogenic mutants, morphological changes were monitored over time using light microscopy. **A.** At an MOI of 1 all strains tested caused epithelial cell rounding (yellow) and detachment (red) within 2–4 h after infection except for the plcR deletion mutant. Intact monolayer (green). **B.** Representative images of Ptk6 cell monolayers. Bar, 20 μm.
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Hybrid cereolysin AB was chosen by Gilmore et al. for the functional unit of phospholipase C (5′-terminal region) and sphingomyelinase (3′-terminal region) proposed to work together as the hemolytic determinant hybrid cereolysin AB of B. cereus with an expected molecular mass of 67 kDa [34]. The peptides identified by mass spectrometry are unique for the 3′-terminal part of hybrid cereolysin AB, which is identical to the sphingomyelinase (SMase) polypeptide. Therefore, since the smaller protein band at approximately 25 kDa is co-detected with the 34 kDa SMase protein band by immunoblot using anti-BCSMase antibody (data not shown), we propose that the 25 kDa protein might be an isoform or degradation product of sphingomyelinase.

Construction of B. cereus sph Gene Deletion Mutant

In order to address the question to which extent SMase contributes to cellular cytotoxicity in vitro, sphingomyelinase null mutants (Δsph) were constructed. Allelic gene replacement of the sph ORF by a chloramphenicol resistance cassette resulted in the B. cereus strains NVH 0075-95 Δsph and NVH 0075-95 ΔsphΔcomP (plc:cm). B. cereus NVH 0075-95 WT and the isogenic mutants ΔahleBC, Δsph and ΔahleBCΔsph grew identical in LB medium at 37°C (Fig. 3A). On sheep blood agar, B. cereus WT showed strong beta-hemolytic activity indicated as clear zones around colonies (Fig. 3B). The hemolysis zone in the nhe mutant was markedly reduced in clearance albeit not in diameter compared to WT, while sph deletion abolished most but not all beta-hemolytic activity. The additional sph deletion resembled a Δplc-like hemolytic-negative phenotype in B. cereus NVH 0075-95 ΔahleBCΔsph suggesting that in HblI, HblII, B. cereus strains like NVH 0075-95 the hemolytic phenotype is conveyed by Nhe and SMase.

Sph Null Mutation does not Affect PC-PLC Activity

Since plc and sph are arranged in one operon (cereolysin AB operon, Fig. 4), the 5′ adjacent plc gene might have been affected by incorrect double crossover during allelic replacement of sph. To test for any polar effects, the mutant strains were cultivated on MYP agar. Typically, phosphatidylcholine-specific phospholipase C (PC-PLC) activity resulted in peripheral zones of egg yolk precipitation on MYP agar (Fig. 3C). This phenotype was not affected by inactivation of nhe or sph. In order to quantify phosphatidylcholine-specific phospholipase C activity of WT and of isogenic sph null strains we determined the lipolytic activity of culture supernatants using a substrate-specific assay for PC-PLC. To correct for putative differences in protein concentrations of the strain specific secretomes, enzyme activities were normalized to the protein content of each sample. Supernatant samples were taken from cells at OD₆₀₀= 4, when PC-PLC activity peaked during growth in LB medium at 37°C (data not shown). With 121.6±25.6 mU/µg protein and 120.5±25.2 mU/µg protein, respectively, phospholipolytic activity of B. cereus NVH 0075-95 WT and ΔahleBC was almost identical. After sph deletion PC-PLC activity was still high for B. cereus NVH 0075-95 Δsph (173.1±12.8 mU/µg protein) and ΔahleBCΔsph (169.9±28.4 mU/µg protein) and not significantly different from WT (P=0.146) and ΔahleBC (P=0.271), respectively.

Sph is co-transcribed with plc from a Common Promoter

For complementation of sph null mutants we sought to reconstitute SMase protein expression under its own sph promoter in both sph knockout strains. Yamada et al. [34] and Pomerantsev et al. [35] have described a putative promoter region Pψph directly upstream of the sph start codon, although sph and plc are known to be arranged in one operon (cereolysin AB operon) probably transcribed from the common promoter Pψplc-sph (Fig. 4).

Because of this discrepancy, we used reverse transcriptase PCR to confirm that the plc-sph gene cluster was co-transcribed from the operon promoter region Pψplc-sph (data not shown). As expected from co-transcription analysis the putative promoter region Pψph directly upstream of the sph start codon failed to reconstitute SMase expression in B. cereus NVH 0075-95 Δsph comPψph and ΔahleBCΔsph comPψph (Fig. S2C).

Therefore, in trans complementation of sph null mutants was achieved by introducing the plasmid pAD/ψplc/ψph/psi/ret driving sph expression from the operon promoter Pψplc-sph. SMase expression in complemented strains Δsph comPψplc and ΔahleBCΔsph comPψplc was confirmed by immunoblotting (Fig. S2C). Re-introduction of sph restored beta-hemolytic enzyme activity, while lipolytic activity was not affected (Fig. S2A and S2B). SMase enzyme activity was significantly enhanced in complemented strains restoring wild type activity by 321–558% due to the multicopy nature of the complementation vector pAD (Fig. 5).

SMase adds to Nhe Induced B. cereus Cytotoxicity in vitro

To elucidate the role of SMase to cellular cytotoxicity in vitro, we tested sph deletion mutants in our initial screen for colon epithelial cell cytotoxicity based on the morphological characteristics cell rounding and detachment. This analysis revealed that SMase as a single factor contributed little to B. cereus cytotoxicity compared to WT (Δsph). However, SMase had a significant effect on IEC cytotoxicity in combination with Nhe (ΔahleBCΔsph). Re-expression of SMase in complemented strains Δsph comPψplc and ΔahleBCΔsph comPψplc reversed the effect (Fig. 6A).

In order to quantify the influence of SMase on cell cytotoxicity, polarized IEC were incubated for 4 h with serial dilutions of bacterial supernatant of B. cereus WT and isogenic mutant strains (Fig. 6B). After staining for dead epithelial cells using Propidium iodide (PI), PI positive and total cell numbers were counted using flow cytometry. Mock (LB medium, 1:2) treated IEC had 1.5±0.1 PI positive cells. Undiluted supernatants of B. cereus NVH 0075-95 WT and sph deletion mutant showed high cytotoxicity against IEC resulting in complete detachment and disintegration of epithelial cells rendering them inaccessible for flow cytometric analysis. Wild type cytotoxicity did not drop below 50% before a dilution of 1:16 (Fig. 6B). Deletion of sph significantly reduced B. cereus cytotoxicity compared to WT. PI positive cells decreased to 5.0%±0.5 in B. cereus NVH 0075-95 Δsph at a 1:8 dilution compared to 72.4%±7.8 in WT.

Treatment of epithelial cells with supernatant of B. cereus NVH 0075-95 ΔahleBC mutant strain resulted in 73.0±5.0 PI positive cells for undiluted and 14.7%±2.7 for 1:2 diluted samples. The additional sph deletion reduced significantly the number of dead cells to 3.6±0.6 in B. cereus NVH 0075-95 ΔahleBCΔsph (P<0.05) (Fig. 6B). Our results emphasize the dominant role of the non-hemolytic enterotoxin Nhe for B. cereus cytotoxicity against polarized intestinal epithelial cells and at the same time hint to a separate effect of SMase adding to Nhe cytotoxicity.

SMase Supplements Nhe Cytotoxicity in vitro

To analyze the concerted action of SMase and Nhe for epithelial cell cytotoxicity, we treated polarized IECs with subtoxic dilutions (1:16) of supernatants of B. cereus NVH 0075-95 WT and mutant strains and compared the cytotoxic effect of added purified recombinant SMase. Addition of 0.05, 0.1 and 0.2 U/ml SMase to supernatant of B. cereus NVH 0075-95 ΔahleBCΔsph consistently increased its cytotoxicity against IECs: 0.2 U/ml SMase increased cell death by 5.4-fold (14.5%±0.5 with compared to 2.7%±0.4 without (P<0.0001); Fig. 6C). In contrast when added to supernatant of B. cereus NVH 0075-95 Δsph, the addition of
Figure 2. Identification of cytotoxic protein from *B. cereus* supernatant. A. Bacterial supernatant of *B. cereus* NVH 0075-95 ΔnheBC was separated on a Superdex-75 10/300 GL gel filtration column. Chromatogram of fractionated bacterial proteins is shown (fraction 1–24). Protein fractions were tested on Ptk6 cells for cytotoxicity as described in Experimental procedures. Protein fractions obtained from gel filtration were analyzed by SDS-PAGE. B. Gel filtration fractions 12–14 transferred cytotoxicity to Ptk6 cells and contained two distinct proteins migrating at 34 kDa and 25 kDa (red asterisks). C. Comparing total extracellular proteins of WT and mutant *B. cereus* strains, two potential cytotoxic proteins (red asterisks) were absent in the supernatant of avirulent ΔplcR strain. doi:10.1371/journal.pone.0061404.g002
0.05, 0.1 and 0.2 U/ml SMase caused a much greater increase in cytotoxicity in the presence of a non-toxic level of Nhe in the supernatant of \( D_{\text{sph}} \): 0.2 U/ml SMase increased cytotoxicity by 14.4-fold (67.6%\( \pm \)15.6 with compared to 4.7%\( \pm \)0.6 without (\( P<0.001 \); Fig. 6C).

These results suggest a cooperative mechanism of enterotoxin Nhe and SMase activity in epithelial cell death in vitro. To ensure that the increase in cytotoxicity after addition of SMase was due to the remaining Nhe activity in the supernatant of \( D_{\text{nheBC/sph}} \), we blocked Nhe activity by using a monoclonal anti-NheB antibody (MAb 1E11) [27], which has been shown to block Nhe activity [45], in the supernatant of the \( sph \) deletion strain. Neutralization of Nhe activity resulted in a strong decrease of SMase cell cytotoxicity in a way similar to the double-knockout \( D_{\text{nheBC}} \)Sph (Fig. 6C) revealing the synergistic effect of Nhe and SMase.

To confirm the synergy of Nhe and SMase proteins a test similar to the classical Christie, Atkins, Munch-Peterson (CAMP)
We could not detect statistical differences between colony-forming units of WT or mutant strains within living (P>0.05) or dead larvae (P>0.05) indicating that in our study reduced pathogenicity associated with sph deletion was not caused by differences in growth in vivo.

Our study shows that B. cereus SMase is an important virulence factor for in vivo pathogenesis in non-gastrointestinal-tract infections. Furthermore, it demonstrates a cooperative interaction of the major enterotoxin Nhe and phospholipase SMase in B. cereus virulence.

**Discussion**

*B. cereus* infections in humans are usually mild causing brief episodes of gastrointestinal symptoms and are probably highly underreported [4]. Considering the strong cytotoxic effect on eukaryotic cells in cell culture experiments in vitro it is fortunate that the bacteria must lack certain qualities that e.g. their close relative *B. anthracis* seem to possess. Even *B. cereus* strains isolated from probiotic formulas are cytotoxic to eukaryotic cells in vitro at a very low MOI of 1, whereas usually high doses of $10^7$ to $10^8$ enterotoxigenic *B. cereus* cells or spores are required for food poisoning [4, 47]. Once the protective barrier of the mucosa or dermis is overcome, *B. cereus* causes serious and often-fatal non-gastrointestinal-tract infections characterized by extensive tissue destruction [3]. Although *B. cereus* infections in the past have been rarely detected as the causative agent of infectious diseases, recent events demonstrate that new highly virulent pathogens can emerge due to the plasticity of bacterial genomes causing outbreaks with serious outcomes for affected patients [48].

Much of *B. cereus* virulence is mediated by secreted enterotoxins. Three cytotoxins, CytK, Hbl and Nhe, are currently considered to be responsible for *B. cereus* diarrhoeal disease but of those three only Nhe is present in most *B. cereus* strains currently known [4]. In contrast, enterotoxin genes *hbl* and *cytK* are often absent even from *B. cereus* strains isolated from disease outbreaks, which argues against a prominent role of these two toxins in disease formation [22, 23]. Nhe is considered to be the major factor in *B. cereus* diarrhoeal disease, because in vitro *B. cereus* cytotoxicity correlates well with Nhe concentration in the bacterial supernatant [49]. Neutralizing antibodies against Nhe abolished almost completely cytotoxicity and a *B. cereus nheBC* mutant NVH 0075-95 lost all cytotoxicity in cell culture based assays [27, 44, 49].

In our study we used the previously described *B. cereus* Nhe mutant strain NVH 0075-95 *ΔnheBC* [44] to identify *B. cereus* sphingomyelinase as an additional virulence factor. Incubation of polarized colon epithelial cells (PK6) with the supernatant of the *ΔnheBC* mutant strain for 4 h resulted in nearly 90% of dead cells revealing that other secreted cytotoxins must contribute to epithelial cell cytotoxicity as well. A major difference to the previous study, in which the lack of Nhe in *B. cereus* NVH 0075-95 (*ΔnheBC*) abolished the entire cytotoxicity of the wild type strain, was time of incubation [44]. Fagerlund *et al.* described that the uptake of Propidium iodide into Vero and Caco-2 cells as an indicator for cell death was abolished in the absence of Nhe in the first 15–30 minutes after adding bacterial supernatant [44]. Our observation that the onset of cell rounding and detachment was delayed in *B. cereus* NVH 0075-95 *ΔnheBC* compared to WT supports the finding that Nhe plays a significant role in the early phase of cell cytotoxicity. However, both strains caused cell death and complete detachment of epithelial cells 4 h after adding bacterial supernatants to cells.

Our study confirmed the pivotal role of the PlcR regulon in *B. cereus* mediated cytotoxicity and we identified sphingomyelinase as...
an important factor contributing to *B. cereus* cytotoxicity. SMase has been described before as a metal ion dependent phospholipase showing hemolytic activity against sheep erythrocytes [50]. The capacity of *B. cereus* to detach host cells in vitro correlated positively with the genomic presence of the *sph* gene [51]. Protein homologues of SMase in *S. aureus* (*β*-toxin) and *Cl. perfringens* (*α*-toxin) are well-established virulence factors involved in cytotoxicity against host cells [17,52]. Walev *et al.* demonstrated cytotoxicity of *S. aureus* *β*-toxin, *Stenotrophomonas sp.* SMase and *B. cereus* SMase against human monocytes [53]. *B. cereus* SMase has been shown to inhibit neurite outgrowth in PC12 cells and to induce membrane damage in host neural cells [54]. However, the role of *B. cereus* SMase as a virulence factor is still under debate, because SMase was nontoxic against Vero cells and in an *in vitro* retinal toxicity model [55,56]. Differences in cytotoxicity observed between various cell lines may be attributed to variations in phospholipid composition and sphingomyelin content of host cell membranes as seen for red blood cells of different species varying in their sphingomyelin content ranging from 25–53.1% (human – sheep) [20,57].

In our current study we demonstrate that SMase is a cytotoxic factor in vitro and its effect is significantly enhanced in cooperation with Nhe. The significance of SMase to overall *B. cereus* pathogenicity was demonstrated in *sph* mutants in wild type and *NVH 0075-95 DnheBC*. In vitro, *sph* deletion alone had little effect on epithelial cell cytotoxicity. But it added significantly to Nhe mediated cell cytotoxicity, suggesting a synergistic mode of action for the pore-forming enterotoxin Nhe and SMase. In the absence of the Nhe membrane-binding components B and C (supernatant

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**Figure 6. Sph deletion effects *B. cereus* virulence *in vitro*.**

**A.** Cytotoxic effect of sterile *B. cereus* supernatants (1:4 diluted) on IEC. Intact monolayer (green), cell rounding <50% (yellow), cell rounding >50% (orange) and 95–100% cell detachment (red) are indicated. **B.** Cytotoxic effects of *B. cereus* supernatant on IEC were analyzed using flow cytometry. Ptk6 cells were treated with various dilutions of bacterial supernatant of *B. cereus* NVH 0075-95 WT (black line), ΔnheBC mutant (grey line), Δsph mutant (black dashed line) and ΔnheBCΔsph mutant (grey dashed line). Samples were stained with Propidium iodide (PI) for dead epithelial cells and cytotoxicity is expressed in % of PI positive cells as determined by flow cytometric analysis. Cytotoxicity of the Δsph mutant was strongly reduced at a dilution of 1:8 compared to WT (a, *P*<0.05). Δsph deletion in addition to Nhe inactivation significantly reduced cytotoxicity compared to Nhe inactivation alone (b, *P*<0.05). Data plotted represent mean values ± SEM (n=3). **C.** Cooperative cytotoxic interaction of SMase and Nhe. Addition of various concentrations of recombinant SMase (0.05, 0.1 and 0.2 U/ml) to diluted (1:16) bacterial supernatants caused significantly higher cytotoxicity against Ptk6 cells when subtoxic Nhe concentrations were present (Δsph supernatant, black line) compared to supernatant without Nhe (ΔnheBCΔsph supernatant, grey line) (*P*<0.05). Addition of anti-NheB (1E11) antibody (10 µg/well) neutralizes Nhe activity (Δsph supernatant+α-NheB, black dotted line). Cytotoxicity is expressed in % of PI positive cells and data represent mean values ± SEM (n=3). **D.** CAMP-like test on sheep blood agar demonstrated complementation of extracellular hemolytic activity between *B. cereus* NVH 0075-95 Δsph and ΔnheBC. Beta-hemolytic activity appeared as cleared zone around the colonies.

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of \(\Delta nheBC\Delta sph\) the addition of recombinant sphingomyelinase resulted in a small but significant increase of epithelial cell cytotoxicity. However, Nhe in the \(B.\ cereus\) supernatant (\(\Delta sph\)), the \(nheBC\Delta sph\) mutant strain (\(\Delta nheBC\Delta sph\)) or its complemented strain (\(\Delta nheBC\Delta sph\) complement) as described in Materials and Methods. Larvae infected with the non-insecticidal \(E.\ coli\) strain DH10B served as control group. \(G.\ mellonella\) survival data are plotted as Kaplan-Meier plots. Data are retrieved from two independent infection experiments with a total of 60 larvae per condition. Both experiments showed very similar results. Statistical significance was determined using log-rank analysis. An asterisk indicates treatment groups with a survival distribution function statistically different from \(B.\ cereus\) WT (\(P < 0.001\)). B. Survival and multiplication of \(B.\ cereus\) WT and isogenic mutant strains in \(G.\ mellonella\) after intrahemocoelic injection of \(10^5\) vegetative cells. Bacterial growth in two independent infection assays was monitored at indicated time points after infection (\(t = 0\)) by counting individual homogenates of five larvae per condition. CFUs recovered from dead larvae are indicated as encircled data points. Paired Student\'s t-test was used to determine statistical differences between bacterial cell counts of the five treatment groups. In living or dead \(Galleria\) larvae cell counts of the \(sph\) mutant strains did not differ significantly from WT and \(\Delta nheBC\) mutant.

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Figure 7. \(Sph\) deletion strongly reduced the pathogenicity of \(B.\ cereus\) NVH 0075-95 in a \(Galleria\) \(mellonella\) in vivo model. A. Larvae were infected by intrahemocoelic injection of \(10^5\) CFU of vegetative \(B.\ cereus\) NVH 0075-95 (WT), the isogenic \(nheBC\) mutant (\(\Delta nheBC\)), the isogenic \(sph\) null mutant (\(\Delta sph\)), the \(nheBC\Delta sph\) mutant strain (\(\Delta nheBC\Delta sph\)) or its complemented strain (\(\Delta nheBC\Delta sph\) complement) as described in Materials and Methods. Larvae infected with the non-insecticidal \(E.\ coli\) strain DH10B served as control group. \(G.\ mellonella\) survival data are plotted as Kaplan-Meier plots. Data are retrieved from two independent infection experiments with a total of 60 larvae per condition. Both experiments showed very similar results. Statistical significance was determined using log-rank analysis. An asterisk indicates treatment groups with a survival distribution function statistically different from \(B.\ cereus\) WT (\(P < 0.001\)). B. Survival and multiplication of \(B.\ cereus\) WT and isogenic mutant strains in \(G.\ mellonella\) after intrahemocoelic injection of \(10^5\) vegetative cells. Bacterial growth in two independent infection assays was monitored at indicated time points after infection (\(t = 0\)) by counting individual homogenates of five larvae per condition. CFUs recovered from dead larvae are indicated as encircled data points. Paired Student\'s t-test was used to determine statistical differences between bacterial cell counts of the five treatment groups. In living or dead \(Galleria\) larvae cell counts of the \(sph\) mutant strains did not differ significantly from WT and \(\Delta nheBC\) mutant.
in a way similar to the double-knockout mutant ΔnheBCΔph. The antibody 1E11 has been demonstrated to inhibit the association of Nhe A with cell-bound NheB via binding to the C-terminal part of NheB, thereby neutralizing Nhe activity [43].

Once the Nhe complex has formed functional transmembrane pores into the epithelial cell membrane, SMase could enter cells more easily hence reach otherwise inaccessible substrate pools in the inner membrane leaflet. SM hydrolysis could result in cell membrane destabilization as well as cell apoptosis via the ceramide-intracellular signaling pathway [60]. Another conceivable mechanism of SMase and Nhe synergism might be that the enzymatic breakdown of membrane sphingomyelin to ceramide by SMase provides access to converted ceramide or other membrane components that can act as binding sites for the active Nhe enterotoxin complex. Such a mechanism has been demonstrated for several bacterial secreted factors such as S. aureus β-toxin and the classical S. agalactiae CAMP factor or Proteus mirabilis and a host acidic SMase which synergistically enhance lysis of erythrocytes [61,62]. The CAMP factor of S. agalactiae has been similar to B. cereus Nhe characterized as an oligomeric pore-forming toxin [63]. Therefore, it is tempting to speculate that B. cereus Nhe could be a CAMP-like factor.

The importance of B. cereus SMase as a virulence factor is strongly supported by in vivo observations in various insects. B. cereus SMase caused significant mortality of German cockroaches, cut- and silkworms when applied as a purified enzyme expressed in B. cereus SMase caused significant mortality of German cockroaches, in vivo. Nhe could be a CAMP-like factor.

The importance of B. cereus SMase as a virulence factor is strongly supported by in vivo observations in various insects. B. cereus SMase caused significant mortality of German cockroaches, cut- and silkworms when applied as a purified enzyme expressed in B. cereus or E. coli [64,65]. Insect models in general and larvae of the great wax moth Galleria mellonella in particular are widely used for the assessment of bacterial pathogenicity in vivo, since the invertebrate immune system functionally resembles the mammalian innate immune response to bacterial infections [8,10,14,66,67]. In the G. mellonella model we demonstrated that sph expression is important for the pathogenic phenotype of B. cereus NVH 0075-95 suggesting that SMase is a significant virulence factor in vitro and in vivo, necessary to establish a severe course of infection. These data confirm recent results published by Oda et al. demonstrating that SMase is essential for B. cereus induced mouse mortality [19]. They showed that SMase mediates bacterial growth of clinical isolates in vivo and evasion from the host innate immune system in contrast to environmental B. cereus isolates. B. cereus strains isolated from soil did not cause lethality in mice due to their lack of SMase, but addition of SMase to B. cereus environmental strains injected into the peritoneum reconstituted virulence of clinical isolates causing them to grow in vivo [19]. However, in G. mellonella SMase may convey mortality in a different way than in mice, because our sph deletion mutants did not show differences in growth.

Given the strong role of Nhe for in vitro cell cytotoxicity, we were surprised to see that the B. cereus NVH 0075-95 ΔnheBC mutant strain behaved similar to wild type in G. mellonella larvae suggesting that Nhe’s sole contribution to B. cereus pathogenicity in vivo is maybe less than what is predicted from in vitro results. The mortality of G. mellonella larvae was significantly reduced in the B. cereus sph deletion mutant. The additional inactivation of nheB/nheC reduced larvae mortality even further, supporting our in vitro observation that SMase and Nhe cooperatively determine B. cereus virulence.

In summary, our data show that the contribution of SMase to B. cereus virulence has been underestimated in the past and our results obtained in cell culture and the Galleria model using deletion mutants of B. cereus confirm that B. cereus SMase contributes in cooperation with Nhe significantly to in vitro cytotoxicity and in vivo pathogenicity. Therefore, it is tempting to speculate that this is also the case in the human host. Secreted factors like SMase and enterotoxins may interact synergistically to cause tissue destructive effects in human B. cereus infections.

Supporting Information
Figure S1 Cytotoxic effects of sterile B. cereus supernatants on IEC. Ptk6 cells were treated with B. cereus F4810/72 and NVH 0075-95 WT and isogenic mutant strains. Morphological changes of Ptk6 cells were monitored over time using light microscopy. All diluted supernatants (1:2) caused immediate epithelial cell rounding and detachment except for the plcR deletion mutant. Intracellular layer (green), cell rounding <50% (yellow), cell rounding >50% (orange) and 100% cell detachment (red) are indicated.

Figure S2 Characterization of sph deletion mutants, complemented and parental B. cereus strains. A. Hemolytic activity of B. cereus WT and isogenic mutant strains on Columbia agar (5% sheep blood, Oxoid). B. Colony morphology of WT and isogenic mutants on MYP (mannitol egg yolk polymyxin) agar indicating PC-PLC enzyme activity. C. Western blot analysis of SMase expression using a polyclonal anti-BcSMase antibody (1:1000). Cells were grown in LB at 37 °C and supernatants were harvested at similar OD600. Identical amounts (4 µg) of total protein preparations were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Lanes: 1, B. cereus NVH 0075-95 (WT); 2, nheB truncation and nheC deletion mutant strain of NVH 0075-95 (ΔnheBC); 3, sph deletion mutant of NVH 0075-95 (Δph); 4, NVH 0075-95 Δph comPph; 5, NVH 0075-95 Δph comPplc; 6, sph deletion complemented via pAD/ph/Pph/tet; 7, NVH 0075-95 Δph comPplc; 8, NVH 0075-95 ΔnheBCΔsph comPplc.

Table S1 Bacterial strains used in this study.

Table S2 Plasmids and oligonucleotides used in this study.

Table S3 Characteristics and toxin gene profiles of B. cereus strains used for cytotoxicity screening.

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Author Contributions
Conceived and designed the experiments: VMD MES RV. Performed the experiments: VMD. Analyzed the data: VMD MES RV. Contributed reagents/materials/analysis tools: VMD MES RV. Wrote the paper: VMD MES RV.
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