Role of Sphingomyelinase in Infectious Diseases Caused by Bacillus cereus

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Abstract

Bacillus cereus (B. cereus) is a pathogen in opportunistic infections. Here we show that Bacillus cereus sphingomyelinase (BcSMase) is a virulence factor for septicemia. Clinical isolates produced large amounts of Bc-SMase, grew in vivo, and caused death among mice, but ATCC strains isolated from soil did not. A transformant of the ATCC strain carrying a recombinant plasmid containing the Bc-SMase gene grew in vivo, but that with the gene for E53A, which has little enzymatic activity, did not. Administration of an anti-Bc-SMase antibody and immunization against Bc-SMase prevented death caused by the clinical isolates, showing that Bc-SMase plays an important role in the diseases caused by B. cereus. Treatment of mouse macrophages with Bc-SMase resulted in a reduction in the generation of H2O2 and phagocytosis of macrophages induced by peptidoglycan (PGN), but no effect on the release of TNF-α and little release of LDH under our experimental conditions. Confocal laser microscopy showed that the treatment of mouse macrophages with Bc-SMase resulted in the formation of ceramide-rich domains. A photobleaching analysis suggested that the cells treated with Bc-SMase exhibited a reduction in membrane fluidity. The results suggest that Bc-SMase is essential for the hydrolysis of SM in membranes, leading to a reduction in phagocytosis.

Introduction

B. cereus is well-known for its role as a mediator of food-borne illness [1,2,3,4]. The microorganism, which forms spores, is ubiquitous in the hospital environment, indicating that contamination of dressings, intravenous catheters, and linen provides an opportunity for infection [5]. It is possible that B. cereus is a pathogen of nosocomial infections transmitted via towels, linen, and balloons to compromised patients [6,7]. In recent years, there has been an increasing appreciation for its potential as an opportunistic pathogen in immunocompromised hosts [5,6,8,9]. The microorganism secretes a wide variety of membrane-damaging toxins, phospholipases such as Bc-SMase, phosphatidylinositol-specific phospholipase C (PIPLC) and phosphatidylcholine-specific phospholipase C (PCLPC), and hemolysins such as cereolysin O, hemolysins and proteases [3,10,11,12,13,14]. However, there has been little research into the contributions of these enzymes and toxins to the infectious diseases caused by B. cereus.

The SMase produced by the intracellular pathogen Listeria ivanovii was shown to mediate bacterial escape from the phagocytic vacuole following internalization, thereby promoting intracellular survival and propagation [15]. Helicobacter pylori-derived SMase was found to contribute toward cytotoxicity for gastric cells [16]. β-Hemolysin containing SMase activity from methicillin-resistant Staphylococcus aureus was expressed by 91% of strains in a high-toxicity group [17]. A mutant strain with deletions of β-hemolysin and catalase was significantly less virulent to mice than the wild-type Staphylococcus aureus strain [18]. We reported that Bc-SMase lysed sheep erythrocytes containing large amounts of SM in the outer lipid layer of their plasma membranes [19]. However, the enzyme is known not to be lethal or cytotoxic. Bc-SMase belongs to a family of Mg2+-dependent neutral SMases (nSMase) that includes SMases produced by Staphylococcus aureus, and Listeria ivanovii [20]. The members of this family share a high degree of homology in amino acid sequence [20,21,22]. However, the role of Bc-SMase in the virulence of B. cereus remains controversial.

To investigate the relationship between Bc-SMase and B. cereus infectious, we examined the relationship between Bc-SMase and the growth in vivo of clinical isolates of B. cereus.
Results

Pathogenicity of the Clinical Isolates of *B. cereus*

To investigate if *B. cereus* JMU-06B-31 and JMU-06B-1, isolated from a patient with septicemia, and JMU-06B-35, isolated from a patient with endophthalmitis, grow in mice in vivo, six-to eight-week-old male wild-type mice of the ICR mice were each injected intraperitoneally with 5 × 10⁸ CFU of the clinical isolates or ATCC21928, ATCC31429, and ATCC6464 isolated from soil. Mice administered with the clinical isolates began to die after 12 h, and all mice died within 30 h of the administration (Fig. 1A). The number of microorganisms in the blood of mice about 12 h after the administration of JMU-06B-31, JMU-06B-35, and JMU-06B-1 was 300–400 CFU/100 μL, whereas the ATCC strains were not detected in blood (Fig. 1B).

Production of Phospholipases by the Clinical Isolates and the ATCC Strains of *B. cereus*

Phospholipases produced by bacteria such as *Staphylococcus aureus*, *Clostridium perfringens*, and *Helicobacter pylori* are reported to be associated with local infections and of importance in the establishment of systemic diseases [4,16,23,24]. To analyze the production of phospholipases by *B. cereus*, we measured the amount of phospholipases produced by the clinical isolates and the ATCC strains in Luria Broth medium. These strains were cultured to an optical density at 620 nm of 0.8 in the medium. The enzyme samples fractionated from the culture supernatants were subjected to SDS-PAGE and Western blotting using anti-Be-SMase, -PCPLC, and -PIPLC antibodies. As shown in Fig. 2A, large amounts (>5 μg/ml) of Be-SMase, PCPLC, and PIPLC were detected in the culture supernatants of the clinical isolates, but very small amounts or undetectable levels in those of the ATCC strains. These phospholipase C genes were detected in every clinical and ATCC strain (Figure S1).

Next, we focused on the promoter sequence for the *Bc-SMase* gene (*smase*) or *PLC* gene (*plc*) from clinical isolates and ATCC isolates. The −35 and −10 promoter sequences of *smase* or *plc* from clinical isolates were almost the same as those of ATCC strains (Fig. 2B and 2C). In *B. cereus*, the transcriptional regulator PhcR (Phospholipase C regulator) controls most known virulence factors [25,26], and activates gene expression by binding to a nucleotideic sequence called the ‘PcrR box’ [25]. As shown in Fig. 2B and 2C, there was no clear difference in the sequence of the PhcR box between clinical isolates and ATCC strains. In addition, the amino acid sequence of Be-SMase was highly conserved in all strains (Figure S2).

Effect of Anti-phospholipases on Growth of *B. cereus* in Mice

To provide clues regarding the growth of *B. cereus* in vivo, the effect of anti-phospholipases on the growth of JMU-06B-35 in mice was investigated. Mice were intraperitoneally injected with the clinical isolate (JMU-06B-35, 5 × 10⁸ CFU) 2 h after the intraperitoneal administration of 50 μg of anti-PCPLC, -PIPLC, or -SMase antibody. The anti-Be-SMase antibody completely inhibited the growth of JMU-06B-35 in the bloodstream (Fig. 3A). In addition, the mice injected with the anti-Be-SMase antibody did not die within 100 h (Fig. 3B). The administration of the anti-PIPLC and -PCPLC antibodies had no effect on the growth and lethality of JMU-06B-35 in mice (Fig. 3A and 3B). The concentration of these antibodies was enough to neutralize the activity of the three enzymes (10 μg in vitro (data not shown)). It therefore appears that Be-SMase plays an important role in the propagation of *B. cereus* in vivo in our experimental condition.

To confirm the relationship between Be-SMase and the growth of *B. cereus* in vivo, we investigated the effect of immunization of mice with Be-SMase, PCPLC, or PIPLC on the death induced by JMU-06B-35. The BALB/c mice were immunized with 25 μg mixture of PCPLC, PIPLC, or Be-SMase with Complete Freund’s

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Figure 1. Lethal challenges with clinical isolates and ATCC strains of *B. cereus*. Mice were intraperitoneally administered with clinical isolates and ATCC strains of *B. cereus* (3 × 10⁸ CFU/mouse). Clinical isolates: JMU-06B-31 (●), JMU-06B-35 (■), and JMU-06B-1 (▲). ATCC strains: ATCC21928 (□), ATCC31429 (○), and ATCC6464 (△). A) Mice were monitored every five hours after the injection. The duration of the experiment was set at 100 h. B) The microorganisms in the blood of mice about 12 h after the administration of various strains were cultured on Luria Broth agar plates. Values represent the mean ± SEM; n = 5 independent experiments. ND: not detected.

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adjuvant (CFA) two times at two-week intervals. Sham-immunized mice administered the clinical isolate began to die after approximately 10 h, and all mice died within 30 h of the administration (Fig. 3C). The survival rate of mice immunized against Bc-SMase, PCPLC, or PIPLC was 100%, 0%, and 0% 30 h after infection, respectively (Fig. 3C).

Effect of Bc-SMase on Infection of Mice with ATCC Strain Isolated from Soil

To investigate the role of Bc-SMase in B. cereus infections, we examined the effect of Bc-SMase on B. cereus-induced death in mice. The animals were intraperitoneally injected with mixtures of ATCC21928 (5.0×107 CFU/mouse), which did not produce Bc-SMase in the culture supernatants, and various concentrations of Bc-SMase. As shown in Fig. 4A, the increase in the rate of death was dependent on the dose of Bc-SMase above 1.0 μg/mouse. On administration of ATCC21928 and 5.0 μg of Bc-SMase, the death rate was 100% within 30 h (Fig. 4A). Mice injected with ATCC21928 or Bc-SMase alone survived after 100 h under the experimental conditions (Fig. 4A). In addition, the number of microorganisms in blood 12 h after the administration of the mixture of ATCC21928 and 1.0 or 5.0 μg of Bc-SMase was 50–100 and 300–400 CFU/100 ml, respectively (Fig. 4B). On the other hand, the administration of ATCC21928 with PCPLC (5.0 μg/mouse) or PIPLC (5.0 μg/mouse) resulted in no death under the conditions (data not shown).

Figure 2. Expression of phospholipases and promoter sequences of smase from clinical isolates and ATCC strains of B. cereus. A) 50% Ammonium sulfate precipitation fractions of the culture supernatants (1.0 mg protein) were subjected to SDS-PAGE and Western blotting using anti-Bc-SMase, -PCPLC, and -PIPLC antibodies. Lane: 1, JMU-06B-31; 2, JMU-06B-35; 3, JMU-06B-1; 4, ATCC21928; 5, ATCC31429; 6, ATCC6464. A representative result from one of three experiments is shown. B, C) The sequences of the promoter region of plc and smase from clinical isolates and ATCC strains of B. cereus were aligned by the program T-Coffee [44]. Consensus sequences of regulatory elements are indicated in bold type. Gray areas indicate nucleotide sequence differences.

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B

promoter region of plc gene

| Strain   | Sequence                         |
|----------|----------------------------------|
| JMU-06B-1| TATGAGATTTTGCTATTTTTAATTTGAGAATTTCGATCTATAGTTAAGGAATTTCGATCTATAGTTAACCCG |
| JMU-06B-31| TATGAGATTTTGCTATTTTTAATTTGAGAATTTCGATCTATAGTTAAGGAATTTCGATCTATAGTTAACCCG |
| JMU-06B-35| TATGAGATTTTGCTATTTTTAATTTGAGAATTTCGATCTATAGTTAAGGAATTTCGATCTATAGTTAACCCG |
| ATCC21928| TATGAGATTTTGCTATTTTTAATTTGAGAATTTCGATCTATAGTTAAGGAATTTCGATCTATAGTTAACCCG |
| ATCC31429| TATGAGATTTTGCTATTTTTAATTTGAGAATTTCGATCTATAGTTAAGGAATTTCGATCTATAGTTAACCCG |
| ATCC6464| TATGAGATTTTGCTATTTTTAATTTGAGAATTTCGATCTATAGTTAAGGAATTTCGATCTATAGTTAACCCG |

C

promoter region of smase gene

| Strain   | Sequence                         |
|----------|----------------------------------|
| JMU-06B-1| GATTAAAAAAAGGTCAAATCTCAGAGATAAGATACGATTGGCTTTTTATATCTATACA---CATGCAAGATGCAAGT |
| JMU-06B-31| GATTAAAAAAAGGTCAAATCTCAGAGATAAGATACGATTGGCTTTTTATATCTATACA---CATGCAAGATGCAAGT |
| JMU-06B-35| GATTAAAAAAAGGTCAAATCTCAGAGATAAGATACGATTGGCTTTTTATATCTATACA---CATGCAAGATGCAAGT |
| ATCC21928| GATTAAAAAAAGGTCAAATCTCAGAGATAAGATACGATTGGCTTTTTATATCTATACA---CATGCAAGATGCAAGT |
| ATCC31429| GATTAAAAAAAGGTCAAATCTCAGAGATAAGATACGATTGGCTTTTTATATCTATACA---CATGCAAGATGCAAGT |
| ATCC6464| GATTAAAAAAAGGTCAAATCTCAGAGATAAGATACGATTGGCTTTTTATATCTATACA---CATGCAAGATGCAAGT |

Figure 2. Expression of phospholipases and promoter sequences of smase from clinical isolates and ATCC strains of B. cereus. A) 50% Ammonium sulfate precipitation fractions of the culture supernatants (1.0 mg protein) were subjected to SDS-PAGE and Western blotting using anti-Bc-SMase, -PCPLC, and -PIPLC antibodies. Lane: 1, JMU-06B-31; 2, JMU-06B-35; 3, JMU-06B-1; 4, ATCC21928; 5, ATCC31429; 6, ATCC6464. A representative result from one of three experiments is shown. B, C) The sequences of the promoter region of plc and smase from clinical isolates and ATCC strains of B. cereus were aligned by the program T-Coffee [44]. Consensus sequences of regulatory elements are indicated in bold type. Gray areas indicate nucleotide sequence differences.

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Figure 3. Effect of antibody and immunization against Bc-SMase, PCPLC, or PIPLC on lethality of B. cereus. Mice intraperitoneally received 50 μg of anti-SMase, -PCPLC, or -PIPLC antibodies and 2 h after the injection, were intraperitoneally administered B. cereus (JMU-06B-35). A) B. cereus in blood was cultured on Luria Broth agar plates 12 h after the intraperitoneal injection. Values represent the mean ± SEM; n = 3 independent experiments. ND: not detected. B) Mice were monitored every five hours after the injection of B. cereus. The duration of the experiment was set at 100 h. ■, B. cereus; ▲, anti-PIPLC antibody + B. cereus; ●, anti-PCPLC antibody + B. cereus; ○, anti-Bc-SMase antibody + B. cereus. C) Mice subcutaneously received an emulsion of the enzyme (Bc-SMase (▲), PCPLC (●), or PIPLC (▲)) and CFA (■) 2 times every 2 weeks. The immunized mice received B. cereus (JMU-06B-35, 3 × 10⁸ CFU/mouse). The duration of the experiment was set at 100 h.

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Figure 4. Effect of Bc-SMase on the infection with B. cereus or B. subtilis. Mice received various concentrations of Bc-SMase and B. cereus (ATCC21928, 5 × 10⁷ CFU/mouse). A) Mice were monitored every five hours after the injection. The duration of the experiment was set at 100 h. ○, B. cereus; □, 1.0 μg Bc-SMase; △, 5.0 μg Bc-SMase; ▲, 0.1 μg Bc-SMase + B. cereus; ●, 1.0 μg Bc-SMase + B. cereus; ■, 5.0 μg Bc-SMase + B. cereus. B) B. cereus in blood was cultured on Luria broth agar plates. Values represent the mean ± SEM; n = 5 independent experiments.

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Effect of Overexpression of Bc-SMase on Growth of B. cereus or B. subtilis in Mice

To investigate the effect of Bc-SMase on growth of B. cereus in vivo, we transfected a vector expressing smase or the gene for E33A (e53a), a variant which has little enzymatic activity [27] (Table S1), into ATCC21928 or Bacillus subtilis (ISW1215), which did not produce Bc-SMase in the culture supernatants. The ammonium sulfate precipitation fraction of the culture supernatants of these transfected strains was subjected to SDS-PAGE and Western blotting using anti-Bc-SMase antibody. As shown in Fig. 5A, these proteins (>5.0 µg/ml) were detected in the culture supernatants of these transformants carrying smase or e53a, but not in each microorganism transformed with empty vector. When the mice intraperitoneally received ATCC21928 or ISW1215 transformants carrying the smase, the microorganisms were detected about 300–400 CFU/100 ml in bloodstream (Fig. 5B). However, administration of these bacteria carrying e53a had no effect on the growth of each microorganism in vivo (Fig. 5B). The results showed that overexpression of Bc-SMase in ATCC21928 or ISW1215 induced growth of these strains in vivo. In addition, the survival rate 100 h after administration of ATCC21928 transformants carrying the smase was approximately 50%, but that of ISW1215 was 100% (Fig. 5C).

Effect of Bc-SMase on Activation of Macrophages by Peptidoglycan

González Zorn et al. reported that the SMase from Listeria ivanovii mediates bacterial escape from phagocytic cells [15]. The activation of macrophages is known to be related to bactericidal action in vivo. To investigate the effect of Bc-SMase on the activation of macrophages, we assessed the effect of PGN, an activator of macrophages, on macrophages treated with Bc-SMase. Bc-SMase attenuated PGN-activated H2O2 generation and phagocytosis of macrophages in a dose-dependent manner (Fig. 6A and 6B). However, Bc-SMase had no effect on the release of TNF-α induced by PGN from macrophages and induced no release of lactate dehydrogenase (LDH) from the cells (Fig. 6C and 6D). It therefore is likely that Bc-SMase specifically influences H2O2 generation and phagocytosis without impairing membranes of macrophages, suggesting that treatment of macrophages with Bc-SMase results in a change in function of the membranes. It was thought that the frustrated phagocytosis may be dependent on the formation of ceramide in macrophage membranes.

Localization of Ceramide in Membranes of Macrophages Treated with Bc-SMase

To determine the amount of ceramide formed in the macrophages treated with Bc-SMase, macrophages were incubated with Bc-SMase at 37 °C for 30 min. The lipids extracted from the treated cells were phosphorylated by diacylglycerol kinase from Escherichia coli, and developed by reverse-phase thin
layer chromatography (TLC). The level of ceramide in the cells treated with Bc-SMase increased in a dose-dependent manner (Fig. 7A). Using confocal microscopy, Montes et al. found that phospholipase C from P. aeruginosa caused the formation of ceramide-rich domains in biological membranes [28]. We reported that Bc-SMase induced the formation of ceramide-rich domains in membranes of sheep erythrocytes and a decrease in the fluidity of membranes, leading to destabilization under physical stimulation [19]. We investigated whether treatment of macrophages with Bc-SMase results in the local accumulation of BODIPY-ceramide formed in membranes of cells preincubated with BODIPY FL-C12-SM (BODIPY-SM). Fig. 7B (left) shows that the fluorescent substance in membranes of macrophages preincubated with BODIPY-SM was not localized. However, when BODIPY-SM -incubated macrophages were treated with Bc-SMase, the local accumulation of the fluorescent substance was found on membranes of the cells, as shown by the white arrows (Fig. 7B, right). To test whether the site where the substance accumulates coincides with a ceramide-rich site, BODIPY–SM-preincubated membranes treated with Bc-SMase were analyzed using Cy3-labeled anti-ceramide antibody. As shown in Fig. 7C, the distribution of the fluorescence of the antibody was different from that of BODIPY–SM in the untreated cells. In the case of BODIPY–SM-preincubated macrophages treated with Bc-SMase, the location of the fluorescence of Cy3-anti-ceramide antibody was consistent with that of BODIPY, as shown in Fig. 7B, suggesting that BODIPY-ceramide formed from BODIPY-SM in the macrophages treated with Bc-SMase is mostly located in ceramide-rich domains. Klein et al. reported that membrane fluidity of cells was evaluated by measurement of lateral diffusion of fluorescence-labeled SM by FRAP with a confocal laser microscopy [29]. A FRAP analysis revealed that the recovery of effective diffusion for the fluorescence of BODIPY in ceramide-rich domains of the macrophages treated with Bc-SMase decreased to about 70–80%, compared with that of BODIPY-SM in the untreated cells.

Figure 6. Effect of Bc-SMase on activation of mouse macrophages. Mouse macrophages were incubated with or without Bc-SMase at 37°C for 60 min (D), and then treated with PGN (5 μg/ml) for 60 min (A, B, C). H₂O₂ production, phagocytosis, TNF-α release, and LDH release were measured as described in Materials and Methods. Values represent the mean ± SEM; n = 7; * P < 0.01 compared with H₂O₂ production or phagocytosis induced by PGN alone.

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Fig. 7D). It therefore appears that the Bc-SMase-induced formation of ceramide from SM in membranes of macrophages results in a decrease in the fluidity of membranes.

**Discussion**

The present study showed that clinical isolates of *B. cereus*, which produced large amounts of Bc-SMase, from patients with sepsis and endophthalmitis were lethal to mice. The production of Bc-SMase from clinical isolates was greater than that from ATCC strains under our culture conditions. *B. cereus* produces several secreted toxins, the expression of which is controlled by the PlcR [25,26]. A difference in protein level from PlcR regulated proteins has been observed for pathogenic factors such as nonhemolytic enterotoxin and hemolysin BL [30,31]. In addition, variations of the InhA1, NprA, and HlyII, which regulation is independent on PlcR, between pathogenic and nonpathogenic *B. cereus* strains has also been observed [32]. In this study, the sequences of the PlcR box in clinical isolates were almost the same as that in ATCC strains. Therefore, it appears that various factors participate in the pathogenic expression of *B. cereus* and production of Bc-SMase.

Bc-SMase enhanced the growth of *B. cereus* in the peritoneal cavity, and in addition, invaded the bloodstream in mice, causing death. Furthermore, overexpression of Bc-SMase in *B. subtilis*, an avirulent strain, induced growth in mice. The administration of a mixture of ATCC21928 and Bc-SMase resulted in the death of mice, but that of PIPLC or PCPLC did not. In addition, the loss of PlcR-regulated factors, which include Bc-SMase, significantly attenuated the severity of *Bacillus* endophthalmitis [10]. Furthermore, mice administered the anti-Bc-SMase antibody or immunized with Bc-SMase were protected from the lethality of clinical isolates of *B. cereus*. Callegan et al., reported that intraocular infection with wild type *B. cereus* or isogenic mutants specifically deficient in PIPLC or PCPLC resulted in similar degrees of
In conclusion, the hydrolysis of SM to form ceramide in the macrophage membrane treated with Bc-SMase induced the attenuation of membrane fluidity and the frustrated phagocytosis. Bc-SMase plays a crucial role in the evasion from immune response by macrophages during the early stages of infections of B. cereus.

Materials and Methods

Strains

The clinical isolates of B. cereus (JMU-06B-31, JMU-06B-35, and JMU-06B-1) were isolated at Jichi Medical University. These isolates, obtained from patients diagnosed with Bacillus bacteremia or endophthalmitis according to the CDC definition, were identified and characterized as B. cereus, as described previously [6]. The ATCC strains of B. cereus from soil (ATCC21928, ATCC31429, ATCC6464) were purchased from DS Pharma Biomedical, Tokyo, Japan. The characteristics of the isolates were reported previously [6].

Mice

Six- to eight-week old male wild-type mice of the ICR and BALB/c strains (Nihon SLJ, Japan) were used. Experimental protocols were approved by the Institute Animal Care and Use Committee at Tokushima Bunri University. The mice were housed in plastic cage under controlled environmental conditions (temperature 22°C, humidity 55%). Food and water were freely available.

Detection of Genes Encoding Bc-SMase, PCPLC, and PIPLC

The genomic DNA from various strains of B. cereus was extracted with the bacteria genomicPrep Mini Spin kit from GE healthcare (UK). The phospholipase C genes of the genomic DNA were confirmed by PCR using the primer sets described below. Bc-SMase primers were forward, 5’-CAAATGGCCAATCGCT- GAA-3’, reverse, 5’-GGTTTCCTACGTACAGATGGTGTT- GGA-3’. PCPLC primers were forward, 5’-GTATTACAAACGCTTG- CATTTGCTC-3’, reverse, 5’-CAATCGCACGGTTTAGAT- CAATCCATA-3’. PIPLC primers were forward, 5’-ACCTGA- TAGTATACGGTGATAGACGA-3’, reverse, 5’-CGAGCTCACTGTTCCATTTTG-3’.

DNA Cloning and Sequencing

The plK-smase region from B. cereus (JMU-06B-31, JMU-06B-35, and JMU-06B-1, ATCC21928, ATCC31429, ATCC6464) was obtained as a 2.1-kb DNA fragment by PCR using primer sets described below. A1 forward primer: 5’-GTATTACATCGTGATGCTTG-3’, reverse, 5’-GTATTCATCAGTTAAATTTTG-3’. PCPLC primers were forward, 5’-CAATCGCACGGTTTAGAT- CAATCCATA-3’. PIPLC primers were forward, 5’-ACCTGA- TAGTATACGGTGATAGACGA-3’, reverse, 5’-CGAGCTCACTGTTCCATTTTG-3’.

Role of Bacterial Sphingomyelinase

Nucleotide sequencing of the cloned fragments was performed by the dyeoxy chain termination technique with a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, USA) using M13 reverse and forward primers. The genetic sequence was confirmed with an ABI3500 genetic analyzer (Applied Biosystems, USA).

Site-directed Mutagenesis

The transforming site-directed mutagenesis kit (LA PCR in vitro Mutagenesis Kit, Takara, Japan) was used with the primer E53A: 5’-GTATATTTAAATGCGGTTTGAATAGC-3’ to prepare the modified plasmid. The genetic sequence of Bc-SMase in...
each plasmid was confirmed with an ABI310 PRISM™ genetic analyzer (Life technologies, USA).

**Preparation of Bc-SMase and Variants**

Bc-SMase and E53A were overexpressed in *B. subtilis* ISW1214 or *B. cereus* ATCC21928 transformed with the plasmid vector pHY300PLK carrying smase or e53a. The expression and purification of the recombinant Bc-SMase and variants were performed as described previously [21].

**Purification of PCPLC and PIPLC**

PCPLC and PIPLC were overexpressed in *B. subtilis* ISW1214 transformed with pHY300PLK carrying the gene of PCPLC or PIPLC cloned from *B. cereus* IAM1029. The PCPLC and PIPLC were secreted into the culture medium. The 80% (w/v) ammonium sulfate fraction of the Luria Broth was fractionated by chromatography using a Cu²⁺-column and then a DEAE-Sepharose column. The purity of samples was verified using SDS-PAGE, and staining with coomassie Brilliant Blue. The PCPLC and PIPLC were observed as a single band at 28 kDa and 34 kDa, respectively.

**Preparation of Antibodies**

Anti-Bc-SMase, -PCPLC, and -PIPLC antibodies were prepared by immunizing rabbits with 100 μg of the purified phospholipases. CFA (Difco, USA) (1.0 ml) was used to prepare the antiserum. Two hypodermic booster injections were made. Antiserum was obtained 2 weeks after the last injection. Preparation of these antisera was performed using Ab-Rapid PuRe (Protenova, Japan).

**Immunization of Mice**

Inbred 6–8-week old male BALB/c mice were immunized subcutaneously two times at two-week intervals. The immunogens were used 25 μg mixture of PCPLC, PIPLC, or Bc-SMase with CFA.

**ELISA Procedure**

The purified recombinant PCPLC, PIPLC, or Bc-SMase was diluted to 5 μg/ml in a carbonate buffer (0.05, pH 9.5) and used to coat the wells of polystyrene plates (100 μl/well: Nunc-Immuno plates with a Maxisorp surface). The plates were incubated overnight at 4°C, and the next morning washed three times with PBST (PBS/0.05% Tween-20). The remaining sites of absorption were blocked by addition of 200 μl well in PBS containing 3% BSA for 2 h at 37°C. The plates were washed three times with PBST. Serum from each group of immunized animals was serially diluted 2-fold (1:500 to 1:128,000) and examined in triplicate wells (100 μl/well) of the blocked antigen-coated plates and incubated for 1 h at 37°C. The plates were then washed five times with PBST and further incubated at 37°C for 1 h with HRP-conjugated anti-mouse IgG (1:2000). The plates were washed five times with PBST and developed with 100 μl of ortho-phenylenediamine (0.4 mg/ml) in a freshly prepared citrate phosphate buffer (0.1 M, pH5.0) and H₂O₂ (0.4 μg/ml). The reaction was terminated by the addition of 50 μl of 2.5 N H₂SO₄/well. Absorbance was read at 492 nm with a microtiter plate reader.

**Determination of ELISA Titer by Endpoint Dilution**

The serum was diluted 2-fold from 1:500 to 1:128,000, and an absorbance value was determined for each dilution. The cut-off value for the assay was calculated from the reference curve for the control serum. The titer of immune serum was calculated as the reciprocal of the highest dilution yielding a specific optical density above the cut-off value. A significant (P<0.05) value of IgG antibody against recombinant PCPLC, PIPLC, and Bc-SMase was 128,000, 64,000, and 64,000, respectively, when compared with CFA-treated mouse serum.

**Measurement of Cytokines**

The concentration of TNF-α was determined with enzyme-linked absorbent assay kits (R&D systems, USA).

**Culture of Macrophages**

Mouse macrophages were isolated from cells in peritoneal exudates with 2 ml of phenol red-free RPMI1640 medium (Wako Pure Chemical Industries, Japan) supplemented with 5% fetal bovine serum (FBS) (Biowest, USA). After centrifugation at 170×g for 10 min at 4°C, the cell pellet was resuspended in phenol red-free RPMI1640 medium supplemented with 5% FBS. Adherent macrophage monolayers were obtained by plating the cells in 96- or 48-well plastic trays (Falcon, USA).

**Preparation of sheep erythrocytes.** Sheep erythrocytes were suspended in 0.02 M Tris-HCl buffer (pH 7.5) containing 0.9% NaCl (TBS), and centrifuged at 1,100×g for 3 min. The erythrocytes were washed by the centrifugation three times. The number of erythrocytes was determined with a cell counter (Celltac; Nihon Kohden, Japan).

**Determination of Hemolytic Activity**

Bc-SMase and E53A was incubated with sheep erythrocytes (12×10¹⁰ cells/ml) in TBS at 37°C for 30 min, and the cells were chilled at 4°C. The hemolysis of the erythrocytes was measured, as described previously [42]. Hemolysis was expressed as a percentage of the amount of hemoglobin released from 0.1 ml of erythrocytes suspended in 0.4 ml of 0.4% NaCl.

**Preparation of Liposomes**

SM (Nacalai Tesque, Japan) from bovine brain and cholesterol (1:1) in chloroform-methanol (2:1 v/v) were dried with N₂ gas, resuspended in TBS containing 0.1 M calboxyfluoroscein (CF). The liposome suspensions were centrifuged at 22,000×g for 15 min at 4°C to remove the nonencapsulated marker, and washed three times by centrifugation. The resulting liposomes were suspended in 200 μl of TBS.

**The SM-liposome-disruption activity.** The SM-liposome-disruption activity was evaluated at the amount of released-CF in the test aliquot. The SM-liposomes in TBS containing 1 mM MgCl₂ were incubated with Bc-SMase or E53A for 30 min at 37°C. The wavelengths for excitation and measurement were 490 and 530 nm, respectively.

**SMase Activity Assay**

SMase activity was measured using an Amplx Red Sphingomyelinase assay kit (Invitogen, USA).

**Measurement of Intracellular H₂O₂**

Mouse macrophages (80% confluent in 48-well plates) isolated from mouse peritoneal exudates were activated with 5 μg/ml PGN (Sigma, USA) for 60 min in the presence of phenol red-free RPMI1640 medium (supplemented with 5% FBS). H₂O₂ was measured in the supernatants using an H₂O₂ assay kit (Oxis International, USA).
Assay of Phagocytosis

Phagocytic activity was determined by measuring the uptake of fluorescent microspheres (Fluoresbrite Carboxylate Microspheres, 1.75 μm in diameter, Polysciences), as described [34]. Mouse macrophages (80% confluent in 48-well plates) were stimulated by PGN in the presence of 5.0×10^5 fluorescent microspheres per ml. After 3 h incubation, cells were washed, and fluorescent intensity in the cells was determined with a fluorescence imaging analyzer (FLA-1000, Fujifilm, Japan).

Measurement of LDH

LDH activity was determined with LDH assay kits (Wako Pure Chemical Industries, Japan), according to the manufacturer’s instructions.

Determination of Ceramide

Mouse macrophages were incubated with various concentrations of Bc-SMase at 37°C for 60 min in phenol red-free RPMI1640 medium supplemented with 5% FBS. The isolation and the measurement of ceramide were performed as described previously [42,43]. The ceramide, which is from bovine brain, used as stimulants or standard was purchased from Sigma, USA.

Immunofluorescence Staining and Confocal Imaging

Mouse macrophages stained with 2 μM BODIPY-SM were plated on 35-mm glass-bottomed dishes (MatTek, USA). The cells were incubated with Bc-SMase in phenol red-free RPMI medium supplemented with 5% FBS at 37°C for 60 min, and the reaction was stopped by 1.0% paraformaldehyde at room temperature. For antibody labeling, the cells were incubated in PBS containing 4% BSA at room temperature for 60 min, followed by mouse Cy3-labeled-anti-ceramide antibody in PBS for 60 min.

Fluorescence Microscopy

A confocal fluorescence microscope (A1; Nikon, Japan) was used. The excitation wavelength was 488 nm for BODIPY FL C12-SM (Molecular probes, USA). The fluorescence signals were simultaneously collected using NIS-Elements C (Nikon software, Japan) into a channel using bandpass filters of 525/50. The objective lens was used with a zoom factor of 2. The experiments were performed at room temperature.

Fluorescence Recovery after Photobleaching

Fluorescence recovery after photobleaching (FRAP), a technology used to measure the lateral mobility of membranes, was performed with a Nikon A1R confocal laser scanning microscope, according to the manual. Mouse macrophages stained with 2 μM BODIPY-SM were plated on 35-mm glass-bottomed dishes (MatTek, USA). The photobleaching was performed in a 1.5 μm, visually uniform region of the cell membranes. Bleaching was performed with 5% laser intensity for a duration of approximately 1 s (10 scans of the laser) to achieve 20% bleaching of the BODIPY fluorescence. After photobleaching, images were acquired 200 times at 1 s intervals.

Statistics

Results were expressed as the mean ± SEM. n equals the sample size. Statistical comparisons were performed using an unpaired t-test or one-way analysis of variance (ANOVA) with Bonferroni correction. p Values less than 0.05 were considered statistically significant.

Supporting Information

Figure S1 Detection of genes encoding Bc-SMase, PCPLC, and PIPLC. The various strains of B. cereus were determined for mRNA of Bc-SMase, PCPLC, and PIPLC.

Supporting Information

Table S1 Biological activities of E53A. Activity (%) was expressed as the percentage of each activity in the wild-type enzyme. Each value is the mean of five experiments.

Role of Bacterial Sphingomyelinase

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Role of Bacterial Sphingomyelinase