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Cloning, Functional Characterization, and Mode of Action of a Novel Insecticidal Pore-Forming Toxin, Sphaericolysin, Produced by *Bacillus sphaericus*▽

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An insecticidal protein produced by *Bacillus sphaericus* A3-2 was purified to elucidate its structure and mode of action. The active principle purified from the culture broth of A3-2 was a protein with a molecular mass of 53 kDa that rapidly intoxicated German cockroaches (*Blattela germanica*) at a dose of about 100 ng when injected. The insecticidal protein sphaericolysin possessed the undecapeptide motif of cholesterol-dependent cytolysins and had a unique N-terminal sequence. The recombinant protein expressed in *Escherichia coli* was equally as potent as the native protein. Sphaericolysin-induced hemolysis resulted from the protein's pore-forming action. This activity as well as the insecticidal activity was markedly reduced by a Y159A mutation. Also, coapplication of sphaericolysin with cholesterol abolished the insecticidal action, suggesting that cholesterol binding plays an important role in insecticidal activity. Sphaericolysin-lysed neurons dissociated from the thoracic ganglia of the German cockroaches. In addition, sphaericolysin's activity in ganglia was suppressed by the Y159A mutation. The sphaericolysin-induced damage to the cockroach ganglia was greater than the damage to the ganglia of common cutworms (*Spodoptera litura*), which accounts, at least in part, for the higher sensitivity to sphaericolysin displayed by the cockroaches than that displayed by cutworms.

Using entomopathogens as biopesticides can reduce the use of synthetic pesticides. Entomopathogens have been isolated from soils and the carcasses of insects, although the natural resources of the pathogens are not limited to these. The larvae of Myrmeleontidae insects, referred to as ant lions, suck out the body fluids of their prey. Because ant lions can kill prey larger than themselves, it was postulated that they used toxins. From the regurgitated fluid of *Myrmeleon bore* larvae, we have purified an insecticidal protein with a molecular mass of 170 kDa (19) and found that it was produced in the larval region from the thorax to the abdomen (33). In addition to the toxin produced by ant lions, insecticidal proteins were found to be produced by bacterial pathogens isolated from *M. bore* larvae (21, 34). Thus, we have further isolated bacteria from the ant lion's crop, which serves as a reservoir of regurgitated fluid, to evaluate their toxicity to insects. It was found that some bacterial species obtained from the crop exhibit insecticidal actions against *Spodoptera litura* cutworms when injected (22). Of these, the A3-2 isolate was closely related to *Bacillus sphaericus* subgroup IIA on the basis of 16S rRNA gene sequencing and DNA-DNA hybridization tests. This *Bacillus* species is currently used as a biopesticide with mosquitocidal action involving protein toxins, notably, binary toxin, Mtx1, and Mtx2 (2, 6). However, it is not clear whether A3-2 uses such mosquitocidal toxins to kill insects other than mosquitoes.

The aim of this study is to determine an insecticidal factor produced by A3-2 and elucidate its mode of action. Here we report that the active principle responsible for killing cockroaches and cutworms is not the mosquitocidal toxins but a novel toxin possessing a unique N-terminal sequence and the cholesterol-binding cytolysin-like domain. Consistent with this, the toxin rapidly lysed not only hemocytes but also insect neurons, which accounts, at least in part, for its insecticidal activity.

MATERIALS AND METHODS

Bacteria and insects. The bacterial isolate A3-2 from the crops of *Myrmeleon bore* ant lions employed in this study has been deposited in the National Institute of Technology and Evaluation Biological Resource Center (NBRC) in Japan and the National Collections of Industrial, Food, and Marine Bacteria (NCIMB) in the United Kingdom (accession numbers NBRC 101285 and NCIMB14133, respectively). *B. sphaericus* subgroup IIA DSM1867 (strain 1593) was purchased from the German National Resource Centre for Biological Material (DSMZ).

The larvae of common house mosquitoes (*Culex pipiens*) and common cutworms (*Spodoptera litura*) were purchased from Sumika Techno Service Co. Ltd. (Hyogo, Japan). Adult male German cockroaches (*Blattela germanica*) were kindly provided by Sumitomo Chemical Co. Ltd. (Hyogo, Japan). All insects were maintained at 25°C and 60% humidity with photoperiod conditions of 12 h of light and 12 h of darkness.

Cell morphology and biochemical properties of *B. sphaericus* A3-2. The cell morphology, motility, and flagellum type of the isolate A3-2 was investigated using a light microscope and a transmission electron microscope. Endospores were stained with malachite green. Gram staining was conducted by employing the conventional method using crystal violet and Lugor solutions. Acid production from carbohydrates and oxidase activity were analyzed using the API 20E and API 50 CH commercial kits (bioMerieux, France) as directed by the manufacturer. Whole-cell fatty acids, as well as prominent quinones of A3-2, were analyzed by TechnoSuruga Co., Ltd. (Shizuoka, Japan).

Analyses of the genes encoding the mosquitocidal toxins produced by *B. sphaericus* subgroup IIA. To investigate whether the bacterial isolate A3-2 possesses the genes encoding the mosquitocidal binary toxin (52 and 41 kDa), Mtx1, and Mtx2. PCR was conducted according to the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 1 min 30 s. The master mix consisted of 1 U of KOD Plus polymerase (Toyoboseki Co., Ltd., Osaka, Japan), 1 mM $MgSO_4$, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 20 ng of template DNA, and 5 pmol of each primer described below. Total genomic DNA of each bacterium was prepared according to the method of Sambrook and Russell (28). The toxin-coding genes were amplified using the

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TABLE 1. Sequences of primers employed in this study

Primer name	Sequence
51 kDa F	5′-CGCTAAATACTACTCCTACAAGCC-3′
51 kDa R	5′-GGATACGATTGTATACCTGCC-3′
42 kDa F	5′-CCCACAGAAGGAAAGTACATTCGC-3′
42 kDa R	5′-CCTAGTAAAGGTTCACTTGC-3′
Mtx1 F	5′-CAAGCTGCTTCACTTACATG-3′
Mtx1 R	5′-GTCCAGTTACATCTTGAGCC-3′
Mtx2 F	5′-GGAGACTAATTGAATTTTCGGTTTCC-3′
Mtx2 R	5′-GCGATGCTGGGCTATGTTCGTTGTTA-3′
F1	5′-CACCAGTCGATATTTCGATTATTGATTCTG-3′
F2	5′-CCTCTACACATACTTTACCTGCAAG-3′
F3	5′-CCCAAGTTCTCGACAATTCACTGGG-3′
F4	5′-GATGGTAGACATATCGCAC-3′
R1	5′-GATGGATTATTAGGTAGTTCAGCACTTAC-3′
R2	5′-GATACTAACTCATCCACAGCTCCAG-3′
R3	5′-GCTTGGTTGGCAAGTTGTAGGGCTC-3′
R4	5′-GAATAAAGAAGGAACTAGGG-3′
TAIL primer	5′-(A/T)GTGNAG(A/T)ANCANAGA-3′
SMS	5′-GATAATCCAACATATGGTAATGTATCTGG-3′
SMA	5′-CCAGATACATTACCATATGTTGGATTATC-3′
VS	5′-GGGAATTCCATATGGAAACACTTGATAC-3′
VA	5′-CCGCTCGAGCTCGATACTGCCTGTTGGG-3′
MS	5′-GCAAGACTACAAGCTTCAGAATCAATGG-3′
MA	5′-CCATTGATTCTGAAGCTTGTAGTCTTGC-3′

primers listed in Table 1. These primers were designed based on the nucleic acid sequence of accession number AJ224477 in the DDBJ/EMBL/GenBank databases and earlier reports (29, 30).

Evaluation of the insecticidal activities of bacteria and a toxic protein of *B. sphaericus* A3-2. The cultures were shaken in 2 ml of soybean casein digest (SCD) broth (pH 6.0) at 37°C for 16 h, after which each test bacterium was harvested by centrifugation (4,000 × *g*, 10 min) and the resultant pellet was washed three times with 0.8% NaCl solution and finally resuspended in 0.8% NaCl solution. To measure the mosquitocidal activity, the fourth-instar larvae of common house mosquitoes were released in 50 ml of 0.8% NaCl solution containing the bacteria at a dose of 10^7 cells ml^{-1}. After 24 h, the dead wrigglers were counted. The insecticidal assays were done in triplicate, employing 30 larvae in each experiment. The 0.8% NaCl solution was employed as the negative control.

The injection assay employing German cockroaches was conducted at each purification step during the course of purifying the toxic factor from the culture broth of *B. sphaericus* A3-2, as reported earlier (21). Briefly, 2 μl of 25 mM potassium phosphate buffer (KPB; pH 7.5) including each test sample at various doses was injected into the abdominal sites of adult male German cockroaches. Five cockroaches were used for each dose of sample, and the symptoms of the cockroaches were observed 5 min after injection. The minimum dose at which at least four of five insects were paralyzed was determined as the toxicity index of the sample (minimum paralysis dose [MPD], ng/insect). To determine the minimum lethal dose of sphaericolysin against caterpillars, the recombinant protein was injected into the fourth-instar larvae of cutworms at various doses (5 μl). Injection of KPB into the cockroaches and cutworms did not influence their behavior.

Purification of a toxic protein produced by A3-2. The strain A3-2 was precultured aerobically in 2 ml of SCD broth at 25°C for 20 h. Then, 200 ml of SCD medium (pH 6.0) was inoculated with the preculture (total amount of culture broth, 2,000 ml). After being shaken for 16 h at 25°C, the broth was centrifuged at 10,000 × *g* for 20 min at 4°C and the supernatant was filtered using a bottle top vacuum filtration system (Iwaki Glass Co. Ltd., MA). To prevent the proteolytic degradation of the toxic factors, EDTA and pepstatin were added to the filtrate at concentrations of 1 mM and 1 μM, respectively, and then ammonium sulfate was added to the supernatant to give a final concentration of 50% saturation. The resultant precipitate was left on ice for 60 min, harvested by centrifugation at 10,000 × *g* for 45 min at 4°C, and dissolved in ca. 30 ml of 25 mM KPB (pH 7.5). The buffer containing ammonium sulfate was replaced by 25 mM KPB (pH 7.5) using a HiPrep desalting column (GE Healthcare UK Ltd., Buckinghamshire, England), and the protein solution was applied to an anion-exchange column containing Q Sepharose resin (HiPrep 16/10 Q; GE Healthcare UK Ltd.) using an ÄKTA explorer 10S system (GE Healthcare UK Ltd.). After the column was washed with 30 ml of 25 mM KPB (pH 7.5), the absorbed proteins were eluted from the resin with 50 ml of KPB containing 300 mM KCl. The elution fraction was concentrated to 850 μl using a Centricon YM-10 apparatus (Millipore Co., MA) and then fractionated by gel filtration using a Superdex 75 HR 10/300 column (GE Healthcare UK Ltd.) with 25 mM KPB (pH 7.5). A fraction at elution volumes from 13 to 15 ml was collected and applied to a Mono-Q column (GE Healthcare UK Ltd.). The column was washed with 30 ml of 25 mM KPB (pH 7.5), and the absorbed proteins were eluted from the resin by increasing the KCl concentration in KPB linearly from 0 to 300 mM. A single peak was collected as the enriched insecticidal fraction.

At all stages of purification, proteins were detected by their absorbance at 280 nm. The protein concentration of each solution was determined by the Bradford method (5) using Coomassie Plus-200 protein assay reagent (Pierce, IL) with bovine serum albumin as the standard. The purity of the protein sample was checked by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to the method of Laemmli (16) and by staining with Coomassie brilliant blue R250 (Nacalai Tesque Inc., Kyoto, Japan).

Amino acid sequencing of the proteinaceous toxin produced by the A3-2 strain. The N-terminal and internal amino acid sequences of the purified toxic protein were analyzed by Edman degradation. Eighteen amino acid residues at the N-terminal position were determined after the protein sample was blotted onto a polyvinylidene difluoride (PVDF) membrane filter (Immobilon-P transfer membrane; Millipore Co., MA) by using a blotting apparatus (HorizBlot, model AE-6677; ATTO Co., Tokyo, Japan). For analysis of the internal amino acid sequence, 20 amino acid residues at the N termini of two peptide fragments obtained by HPLC after tryptic digestion of the protein were determined by Edman sequencing.

Cloning and sequencing of the insecticidal toxin gene of the A3-2 strain. A part of the toxin-coding gene was amplified by PCR using 10 pmol of the forward primer F1 and the reverse primer R1, both of which were designed on the basis of the perfringolysin-encoding gene of *Bacillus cereus* ATCC 14579 (NCBI database accession no. AE017014 [14]). In addition to primers, the PCR mix contained 0.5 U of LA-*Taq* polymerase (Takara, Japan), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 100 ng of the genomic DNA of A3-2 as the template and was subjected to the following program, repeated 30 times: 94°C for 30 s, 45°C for 1 min, and 72°C for 1.5 min. The resulting amplicon was sequenced directly by the dye terminator method using DYEnamic ET Terminator cycle sequencing kit (GE Healthcare UK Ltd.) in combination with an ABI3100 genetic analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan).

The 5′ and 3′ ends of the toxin gene were obtained by employing thermal asymmetric interlaced PCR (TAIL-PCR) (18) using the forward primers F2 and F3 in combination with the reverse primers R2 and R3 and a degenerate primer as a nonspecific arbitrary primer (TAIL primer). The amplified tertiary TAIL-PCR products were cloned into the pGEM T-easy cloning vector (Promega) according to the manual. Then, the cloned gene was sequenced using the dye terminator method from the T7 and SP6 regions on the pGEM T-easy vector (Promega). Finally, a DNA region containing the entire 53-kDa toxin gene was amplified by PCR using 1 U of KOD Plus polymerase, two primers (F4 and R4), 1 mM $MgSO_4$, 0.2 mM of each dNTP, and 100 ng of the genomic DNA as the template. The following protocol was used: 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 1.5 min. The amplified gene was cloned into the pCRScript Amp SK(+) cloning vector (Stratagene, TX) and sequenced using the dye terminator method.

Functional expression of the insecticidal toxin in *Escherichia coli*. To generate a silent mutation at the NdeI site in the gene of the 53-kDa protein (positions 467 to 472), silent mutation sense (SMS) and silent mutation antisense (SMA) primers were designed. Using these primers together with vector sense (VS) and vector antisense (VA) primers, first-round PCR was performed using 1 U of KOD Plus polymerase, 100 ng of the 53-kDa-toxin gene cloned into the pCRScript vector as the template, 15 pmol of the primers (the primer sets employed were VS and SMA primers and SMS and VA primers, respectively), 1 mM $MgSO_4$, and 0.2 mM of each dNTP in a 50-μl solution for 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 1.5 min. The second-round PCR was performed using 1 U of KOD Plus polymerase, 50 ng each of the first-round PCR products, 15 pmol of the primers (VS and VA primers), 1 mM $MgSO_4$, 0.2 mM of each dNTP in a 50-μl solution for 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 1.5 min, yielding a single band of the predicted size. The PCR product was digested using NdeI and XhoI for 18 h at 37°C and cloned into the NdeI and XhoI sites of the pET 22b (+) vector (Novagen Inc., WI). *E. coli* BL21(DE3) (Novagen Inc., WI) transformed with this expression vector containing the 53-kDa-toxin gene was incubated at 37°C for 3 h, and protein expression was then induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside. After incubation at 25°C for 14 h, *E. coli* cells overexpressing the toxin were lysed with 10 ml of Bugbuster reagent (Novagen Inc., WI) containing 250 U of Benzonase nuclease (Novagen Inc., WI). The supernatant of the bacterial lysate was diluted twofold

with 50 mM KPB (pH 7.5) and applied to a Ni-nitrilotriacetic acid (NTA) affinity column (Ni-NTA His-Bind resin; Novagen Inc., WI). The column was washed with 25 mM KPB (pH 7.5), and the absorbed protein was eluted with 25 mM KPB (pH 7.5) containing 200 mM imidazole. The eluted sample was further purified by gel filtration using a Superdex 75 10/300 column (GE Healthcare UK Ltd.) with 25 mM KPB (pH 7.5).

Site-directed mutagenesis. The mutation Tyr159Ala (Y159A) was introduced by PCR. Mutagenesis sense (MS) and mutagenesis antisense (MA) primers were designed to generate the mutation. A pair of first-round PCRs were carried out using 1 U of KOD Plus polymerase, 100 ng of the wild-type toxin gene cloned into the pET-22b (+) vector as a template, 15 pmol of the primers (VS and MA primers and MS and VA primers, respectively), 1 mM $MgSO_4$, and 0.2 mM of each dNTP in a 50-μl solution for 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 53°C for 30 s, and 68°C for 1.5 min. The second-round PCR was performed using 1 U of KOD Plus polymerase, 70 ng each of the first-round PCR products, 15 pmol of the primers (VS and VA primers), 1 mM $MgSO_4$, and 0.2 mM of each dNTP in a 50-μl solution at 94°C for 2 min followed by 30 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 2 min. The PCR product was purified using the aforementioned method and then digested with NdeI and XhoI and cloned into the NdeI and XhoI sites of the pET-22b (+) vector. The Y159A mutant was expressed in and purified from *E. coli* using the same protocol as that described above.

Pore-forming activity. The pore-forming activity of the wild type and Y159A mutant of the recombinant protein on rat erythrocyte membranes was measured as follows. Erythrocytes were collected from a male Wistar rat (age, 8 weeks; body weight, ca. 300 g; Shimidzu Experiment Materials Co., Kyoto, Japan) and were suspended in a 0.8% aqueous NaCl solution at a concentration of 2×10^7 cells ml^{-1}. Ten microliters of 25 mM KPB (pH 7.5) including a recombinant toxin was added to 140 μl of the erythrocyte suspension; the toxins were used at various concentrations. After incubation at 37°C for 10 min, each suspension was centrifuged at 6,000 × *g* for 5 min at 4°C. The hemoglobin released into the supernatant from erythrocytes lysed by toxins was quantified by determining absorbance at 540 nm. The pore-forming activity was represented as pEC_{50} [= log $(1/EC_{50})$] values, where EC_{50} is half the maximum concentration needed to lyse the cell.

The pore formed on the erythrocyte membrane by the 53-kDa toxin was observed using transmission electron microscopy (TEM). Next, 5 μl of 25 mM KPB (pH 7.5) containing the 53-kDa recombinant protein (448 ng μl^{-1}) was added to 15 μl of the suspension containing erythrocytes at a dose of 3×10^7 cells ml^{-1}, and the suspension was placed onto the grid (sheet mesh, DN 400 mesh; Okenshoji Co. Ltd., Tokyo, Japan) coated with 0.5% Formvar solution (LADD Research, VT). After 30 min, the grid was negatively stained with 2% (wt/vol) phosphotungstic acid (TAAB Laboratories Equipment Ltd., United Kingdom), adjusted to pH 7.2 using 5% (wt/vol) KOH for 20 s, and then observed via TEM (H-800 electron microscope; Hitachi, Ltd., Tokyo, Japan) at an acceleration voltage of 100 kV. Digital pictures were taken with a digital charge-coupled-device camera (Advantage HR; Advanced Microscopy Techniques Co., Danvers, MA), and the pore size was measured using the image analysis software installed in the camera system.

Effects of the 53-kDa toxin on the nervous systems of insects. The third thoracic ganglia of the central nerve cord were dissected from the male adult German cockroaches and from the fourth-instar larvae of the common cutworms. The ganglia were treated with the toxin (50 ng μl^{-1}) for 5 min in Ringer's solution (210 mM NaCl, 2.9 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM Na_2HPO_4, 0.2 mM KH_2PO_4, pH 7.2). Then, the ganglia were stained with trypan blue solution (0.2% in the Ringer's solution) for 10 min to evaluate cellular damage.

To study the effects of the toxin on insect neurons, the third thoracic ganglia from the cockroaches and cutworms were also dissociated as described earlier by Ihara et al. (13). In brief, the third isolated thoracic ganglia were desheathed in saline containing 200 mM NaCl, 3.1 mM KCl, 4 mM $MgCl_2$, and 10 mM HEPES, pH 7.6, and then treated with 1 mg ml^{-1} collagenase (type IA; Sigma) for 1 h at room temperature. Neurons were dissociated by gentle pipetting in the Ca^{2+}-supplemented saline (200 mM NaCl, 3.1 mM KCl, 4 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose, pH 7.4) supplemented with 10% fetal bovine serum. The cockroach and cutworm neurons were placed on a poly-D-lysine-coated cover glass and incubated for 12 to 24 h. The neurons were treated with the 53-kDa toxin (50 ng μl^{-1}), and their morphological changes were microscopically observed 15 min after toxin exposure. Thoracic ganglia and neurons incubated in Ca^{2+}-supplemented saline for 15 min were used as a negative control.

Immunological detection of sphaericolysin in the carcasses of cutworms injected with *B. sphaericus* A3-2. Antiserum for sphaericolysin was raised in a male mouse (5-week-old Jcl:ICR mouse; CLEA Japan, Inc., Tokyo, Japan) that was injected hypodermically with the toxin (60 μg in 300 μl of 0.1% SDS–phosphate-buffered saline [PBS]), which was emulsified with an equivalent volume of complete Freund's adjuvant. The animal received a booster with the same amount of antigen emulsified in incomplete Freund's adjuvant twice every a week. The antiserum prepared from the blood was diluted 2,000-fold with Tris-buffered saline (50 mM Tris-HCl [pH 7.4] including 150 mM NaCl) including 1% (wt/vol) gelatin and 0.05% (vol/vol) Tween 20 prior to use.

After being cultured in 2 ml SCD liquid broth (pH 6.0) with shaking for 16 h at 37°C, the pellet of *B. sphaericus* A3-2 harvested by centrifugation was washed three times with 3 ml 0.8% NaCl solution and then resuspended in 0.8% NaCl solution. The A3-2 suspension was injected into 30 cutworms at a dose of 5×10^5 cells, and the cutworms were left at 25°C for 16 h. The carcasses of the bacterium-injected cutworms were homogenized in 5 ml of PBS. Aliquots of the homogenates were mixed with the same volume of the sample loading buffer (0.075 M Tris-HCl [pH 6.8] containing 5% 2-mercaptoethanol, 2% SDS, 5% sucrose, and 0.004% bromophenol blue) and separated by SDS-PAGE (8%). The proteins were then transferred onto a PVDF membrane using a blotting apparatus. The PVDF membrane was treated with diluted antiserum for sphaericolysin and then reacted with the rabbit anti-mouse immunoglobulin G antibody conjugated with alkaline phosphatase (Chemicon International Inc., Temecula, CA). The secondary antibody was stained with the Western Blue-stabilized substrate for alkaline phosphatase (Promega). Thirty cutworms injected with 0.8% NaCl solution were employed as negative controls. No toxin band was detected in the employed bacterial suspension.

Nucleotide sequence accession number. The nucleotide sequence of the sphaericolysin-encoding gene is available in the DDBJ/EMBL/GenBank databases under the accession number AB273179.

RESULTS

Cell morphology and biochemical properties of *B. sphaericus* A3-2. The microscopic observation of A3-2 indicated that the bacterium is a gram-positive rod occurring both singly and in pairs and occasionally in a short chain. The bacteria were motile and peritrichous, and this isolate had endospores in a cell body. The API test indicated that the A3-2 isolate produces acids from *N*-acetylglucosamine but that it does not produce acids from other carbohydrates. In addition, A3-2 hydrolyzed gelatin and urea. Analyses of quinone using high-performance liquid chromatography indicated that respiratory menaquinone MK-7 (95.8%) was prominent and that MK-6 (4.0%) and MK-8 (0.2%) were minor components, as seen in the genus *Bacillus*. In the analyses of the fatty acid composition, iso-$C_{15:0}$ (56.67%), anteiso-$C_{15:0}$ (11.90%), and $C_{16:1\omega7c}$ alcohol (9.41%) were detected as the major fatty acids. The similarity index estimated using the MIDI database demonstrated that the fatty acid profile of A3-2 was similar to those of *B. sphaericus* GC subgroup IIA (similarity index, 0.578) strains. All the results and our earlier DNA-DNA hybridization test results (the relatedness of A3-2 to DSM1867 was 89% [22]) were in agreement with the data reported earlier (7, 15, 23), suggesting that A3-2 is a strain of *B. sphaericus* subgroup IIA.

***B. sphaericus* subgroup IIA (A3-2) lacks mosquitocidal genes.** Since most of the *B. sphaericus* subgroup IIA species are known to produce the mosquitocidal toxins, we investigated by PCR whether the isolate A3-2 possesses the mosquitocidal toxin genes. It was found that A3-2 lacks mosquitocidal toxin genes (Fig. 1A) but that a type culture, *B. sphaericus* subgroup IIA DSM1867, possesses all of them. Consistent with this, A3-2 had no mosquitocidal activity, whereas *B. sphaericus* subgroup IIA DSM1867 killed approximately 90% of common house mosquitoes at a dose of 10^7 cells ml^{-1} (Fig. 1B).

Purification and amino acid sequencing of the proteinaceous toxin produced by A3-2 strain. The culture broth of

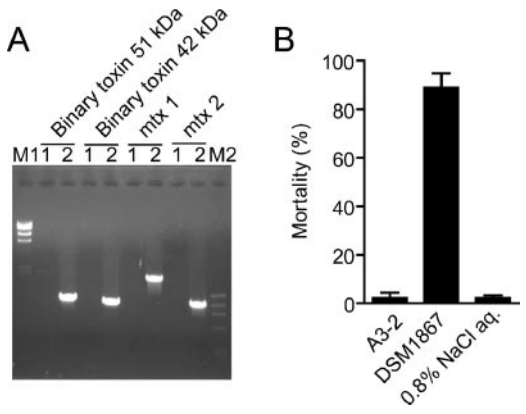

FIG. 1. Insecticidal activity of *Bacillus sphaericus* subgroup IIA. (A) Detection by PCR of the mosquitocidal toxin genes (binary toxin [51 + 42 kDa], Mtx1, and Mtx2). Lanes 1 and 2 contain A3-2 and DSM1867, respectively. Lanes M1 and M2 contain λ-HindIII digest and ϕX174-HaeIII digest markers, respectively. (B) Insecticidal activity toward common house mosquitoes (*Culex pipiens*). The insecticidal assay was performed in triplicate. Each bar and error bar indicate a mean ± SEM. aq., aqueous.

A3-2, which was concentrated using a 10-kDa-cutoff membrane filter, induced rapid paralysis when injected into German cockroaches, although this activity was abolished by heating at 100°C and by proteinase K treatment (data not shown), indicating that the insecticidal factor is proteinaceous. By monitoring the paralytic activity, an insecticidal principle was purified from the culture broth of *B. sphaericus* A3-2 using gel filtration and anion-exchange chromatography (Fig. 2A). In the course of the purification, the insecticidal activity increased about 10-fold in terms of the MPD values (with the 50% ammonium sulfate precipitation purification scheme, the MPD was 613 ng/insect, that with Q Sephacel was 326 ng/insect, that with Superdex 75 was 100 ng/insect, and that with Mono-Q was 58 ng/insect). The purified protein migrated as a single band on 10% SDS-PAGE gels at a molecular mass of 53 kDa (Fig. 2B). The toxin was eluted at a similar molecular mass by gel filtration. The protein intoxicated the German cockroaches within 5 min after injection with an MPD value of 101 ± 38 ng protein per insect (mean ± standard error of the mean [SEM]; four experiments), whereas it did not show rapid toxicity toward the cutworms but killed them after 16 h at a dose of 500 ng per insect ($n = 3$).

The N-terminal amino acid sequence determined by Edman degradation of the toxin (ETLDTNSSSVKSKSDIDT) had no homology to any known proteins, whereas its internal sequences (QIFYTVSAELPNNPSDLFDD and SLTTSPVDISI IDSMANRTY) showed similarities to those of perfringolysin O of *B. cereus* (95% and 85%, respectively). Exploiting the TAIL-PCR method, the entire primary structure of the 53-kDa toxin was determined (Fig. 3). It was found that the toxin consists of 478 amino acid residues with a molecular mass of 53,037.17 Da and is generated from a precursor protein with a molecular mass of 56,118.99 Da by cleavage of the signal peptide. The neural networks and hidden Markov models trained on gram-positive bacteria suggested that the signal peptide cleavage site is located just in front of the N terminus of the 53-kDa protein (SignalP, v.3.0 [3]). A possible start codon of the premature toxin-encoding gene is TTG (leucine), as observed frequently in *B. subtilis* (27). An undecapeptide motif, ECTGLAWEWWR, of the cholesterol-dependent cytolysins is present adjacent to the C terminus (Fig. 3).

Relation of the insecticidal activity of sphaericolysin to cholesterol. The recombinant protein expressed in *E. coli* was purified homogeneously using Ni-NTA affinity chromatography combined with gel filtration chromatography. The insecticidal activity (MPD) of the recombinant protein against the German cockroaches was 55 ± 12 ng/insect (mean ± SEM; $n = 6$), a value similar to that of the native protein.

It has been shown that tyrosine 159 plays an important role in the pore-forming activity of perfringolysin. Thus, the effects of the Y159A mutation on the hemolytic and insecticidal activities of the 53-kDa toxin from A3-2 were investigated. The wild-type toxin lysed erythrocytes clearly, even at a concentration of 1×10^{-14} M (pEC_{50} of 13.87 ± 0.02; $n = 6$), whereas the hemolytic activity of the Y159A mutant was lower, with a pEC_{50} value of 11.77 ± 0.13 ($n = 6$) (Fig. 4). In addition, the Y159A mutation markedly reduced the insecticidal activity against cockroaches (MPD of the Y159A mutant, >860 ng/

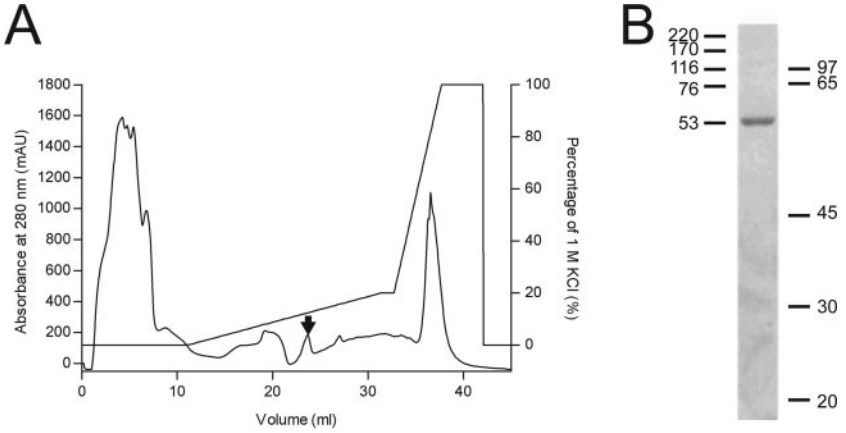

FIG. 2. Purification of the toxic protein produced by isolate A3-2. (A) Anion-exchange chromatography profile of the insecticidal proteins produced by A3-2. The arrow shows the peak collected as the active fraction. (B) SDS-PAGE analysis of the purified toxin. Numbers at the sides of the gel are molecular masses (in kilodaltons).

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      GATGGTAGACATATCGCACAAAATTAACATAATAGTTATCATAATAATCATAAGTACATATTATTTGCACAATAAAAAATGAAACGAAAATACTATATTTG
      AAAATTATTTTTATATGGTATAATTTCCTATAAAAGGAGTGGATTTATTTGGGAATTAAAAAAACAATCAAGTTTATATTATGTTTATCTATTAGTTTATG  53
                                                                  L  G  I  K  K  T  I  K  F  I  L  C  L  S  I  S  L  C
   54 CATTCTAAATTATCCAAGTATTTCATTTGCTGAAACACTTGATACTAATAGTTCTAGTGTAAAAAGCAAATCTGACATTGATACGGGTATAGCAAACTTGA  154
       I  L  N  Y  P  S  I  S  F  A  E  T  L  D  T  N  S  S  S  V  K  S  K  S  D  I  D  T  G  I  A  N  L  N
  155 ACTACAACAACAGGGAAGTACTAGCAGTGAATGGTGACAGAGTTGATAGCTTTGTTCCAAAAGAAGGGCTTAACTCCAATGATAAATTTATAGTGGTGGAG  255
        Y  N  N  R  E  V  L  A  V  N  G  D  R  V  D  S  F  V  P  K  E  G  L  N  S  N  D  K  F  I  V  V  E
  256 CGCAATAAGAAATCACTGACAACTTCACCAGTGGATATATCAATTATTGATTCGATGGCGAATCGTACATATCCAGGAGCCCTACAACTTGCCAACCAAGC  356
       R  N  K  K  S  L  T  T  S  P  V  D  I  S  I  I  D  S  M  A  N  R  T  Y  P  G  A  L  Q  L  A  N  Q  A
  357 TTTTGTAGACAATCAACCCAACTTATTGGTGGCTAAAAGAAAACCCCTAAATATTAGCATTGATTTACCTGGTATGAAAAGAGAAAATACTTTGACTGTTG  457
        F  V  D  N  Q  P  N  L  L  V  A  K  R  K  P  L  N  I  S  I  D  L  P  G  M  K  R  E  N  T  L  T  V  D
  458 ATAATCCAACATATGGTAATGTATCTGGAGCTGTGGATGAGTTAGTATCTACTTGGAGCGAGAAATATTCCTCTACACATACTTTACCTGCAAGACTACAA  558
        N  P  T  Y  G  N  V  S  G  A  V  D  E  L  V  S  T  W  S  E  K  Y  S  S  T  H  T  L  P  A  R  L  Q
  559 TATTCAGAATCAATGGTTTATAGCAAATCTCAAATAGCAAGCGCTTTGAATGTAAACGCCCAAGTTCTCGACAATTCACTGGGAATTGACTTTAATGCGAT  659
       Y  S  E  S  M  V  Y  S  K  S  Q  I  A  S  A  L  N  V  N  A  Q  V  L  D  N  S  L  G  I  D  F  N  A  I
  660 TGCGAATGGAGAGAAAAAAGTGATGGTTGCCGCATATAAACAAATTTTTTATACGGTAAGTGCAGAACTGCCTAACAATCCATCAGATCTTTTCGATGATA  760
        A  N  G  E  K  K  V  M  V  A  A  Y  K  Q  I  F  Y  T  V  S  A  E  L  P  N  N  P  S  D  L  F  D  D  S
  761 GTGTTGATTTTGCAGAGCTAACTCGTAAAGGGGTAAGCAATGATGCTCCTCCTGTAATGGTGTCGAATGTAGCTTATGGTAGAACAATTTATGTGAAATTA  861
        V  D  F  A  E  L  T  R  K  G  V  S  N  D  A  P  P  V  M  V  S  N  V  A  Y  G  R  T  I  Y  V  K  L
  862 GAAACAAGCTCTAAGAGTAAGGATGTACAAGCAGCATTTAAAGCATTACTGAAGAATGTTAACACGAATGTAGAAACTAGTGCACAATACAAGGATATTTT  962
       E  T  S  S  K  S  K  D  V  Q  A  A  F  K  A  L  L  K  N  V  N  T  N  V  E  T  S  A  Q  Y  K  D  I  F
  963 TGAGGAAAGTTCCTTTACCGCTGTAGTATTAGGCGGAGATTCACAAAAGCATAATCAAATTGTCTCAAAGGACTTTAATGATATTAGAGAAGTCATTAAAG 1063
        E  E  S  S  F  T  A  V  V  L  G  G  D  S  Q  K  H  N  Q  I  V  S  K  D  F  N  D  I  R  E  V  I  K  D
 1064 ATAATGGAGAATTTAGTCTTAAAAATCCAGCTTATCCAATTTCCTATACAAGTGTTTTCTTAAAGGATCATTCAATTGCTGCTGTTCATAATAATACAGAT 1164
        N  G  E  F  S  L  K  N  P  A  Y  P  I  S  Y  T  S  V  F  L  K  D  H  S  I  A  A  V  H  N  N  T  D
 1165 TATATTGAGACGACAGCTACAGAATATTCTAAAGGCAAGATCATCCTTGATCATTATGGTGCATACGTTGCTCAATTTGAAATAGCATGGGACGAATTTTC 1265
       Y  I  E  T  T  A  T  E  Y  S  K  G  K  I  I  L  D  H  Y  G  A  Y  V  A  Q  F  E  I  A  W  D  E  F  S
 1266 CTATGATGAGAATGGAAATGAAGTATTAACTCATAAAACGTGGGATGGAAACTGGAGAGATAAAACAGCTCATTTTTCTACAGTCATACCGCTTCCGGCTA 1366
        Y  D  E  N  G  N  E  V  L  T  H  K  T  W  D  G  N  W  R  D  K  T  A  H  F  S  T  V  I  P  L  P  A  N
 1367 ATTCGAAAAATATAAGAATTTATGCAAGAGAATGTACAGGTCTTGCTTGGGAATGGTGGAGAACGGTTATTGATGAATATAATGTTCCATTGTCTAATGAA 1467
        S  K  N  I  R  I  Y  A  R  E  C  T  G  L  A  W  E  W  W  R  T  V  I  D  E  Y  N  V  P  L  S  N  E
 1468 ATCAAAGTCTCCATTGGAGGAACTACATTATACCCAACAGGCAGTATCGAGTAAGTAGCCGTATATAAAGATAAAGAATTTTTACCTCCTAACAAATTAAT 1568
       I  K  V  S  I  G  G  T  T  L  Y  P  T  G  S  I  E  *
 1569 GTTAGGAGGTTTTTACGTTTTCAAGTTCTTTCAATAACTGCCCCATGTTCCTTCTTTATTC  1629
```

FIG. 3. Nucleotide sequence of the gene encoding the 53-kDa toxic protein and its amino acid sequence deduced from the nucleotide sequence. The underlined sequences were determined by Edman degradation as described in the text. The putative Shine-Dalgarno, −10, and −35 sequences are indicated by the dotted lines. The bold line indicates the putative start codon of the precursor protein. An arrowhead indicates a possible cleavage point for the signal peptide that was predicted by employing the neural networks and hidden Markov models trained on sequences in the gram-positive-bacterium database. The NdeI site is indicated as italic characters (positions 467 to 472). A point mutation generated at position 159 is enclosed by a square.

insect; $n = 4$). Since it has been shown that the binding of cholesterol-binding cytolysins (CBCs) to membrane cholesterol is involved in hemolytic activity (1, 4, 8, 32), sphaericolysin was coinjected with cholesterol to examine a possible role of cholesterol in the insecticidal activity of sphaericolysin. It was found that coapplication with 2 μg of cholesterol markedly reduced the insecticidal activity of the recombinant sphaericolysin (482 ± 73 ng/insect) ($n = 4$).

The pore-forming action of the protein on the erythrocyte membrane was evaluated using TEM (Fig. 5). The toxin forms many pores with a diameter of 35 ± 1 nm (mean diameter of six pores measured separately ± SEM), which is close to the diameter of pores formed by the CBCs perfringolysin O and listeriolysin O (1, 4, 8, 32).

Effects of sphaericolysin on the nerve systems of German cockroaches and common cutworms. The nerve cords dis-

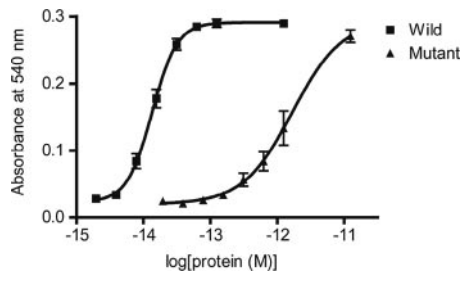

FIG. 4. Hemolysis activity of sphaericolysin produced by A3-2 and its Y159A mutant. Ten microliters of sample solution at each dose was mixed with 140 μl of erythrocyte suspension (2×10^7 cells ml^{-1}), and the quantity of hemoglobin released from erythrocytes into the supernatant was detected by absorbance at 540 nm. Each plot indicates means ± SEMs ($n = 4$ to 6).

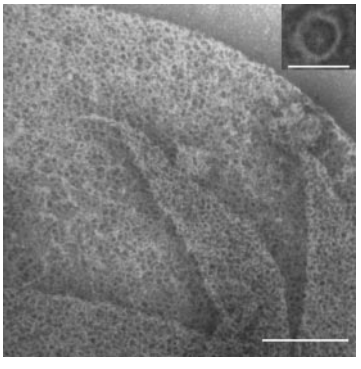

FIG. 5. Pore formation by sphaericolysin on the membranes of erythrocytes as observed with a transmission electron microscope using the negative-staining method. Magnification, ×50,000; scale bar, 500 nm. The inset is a magnified picture of a single pore (scale bar, 50 nm).

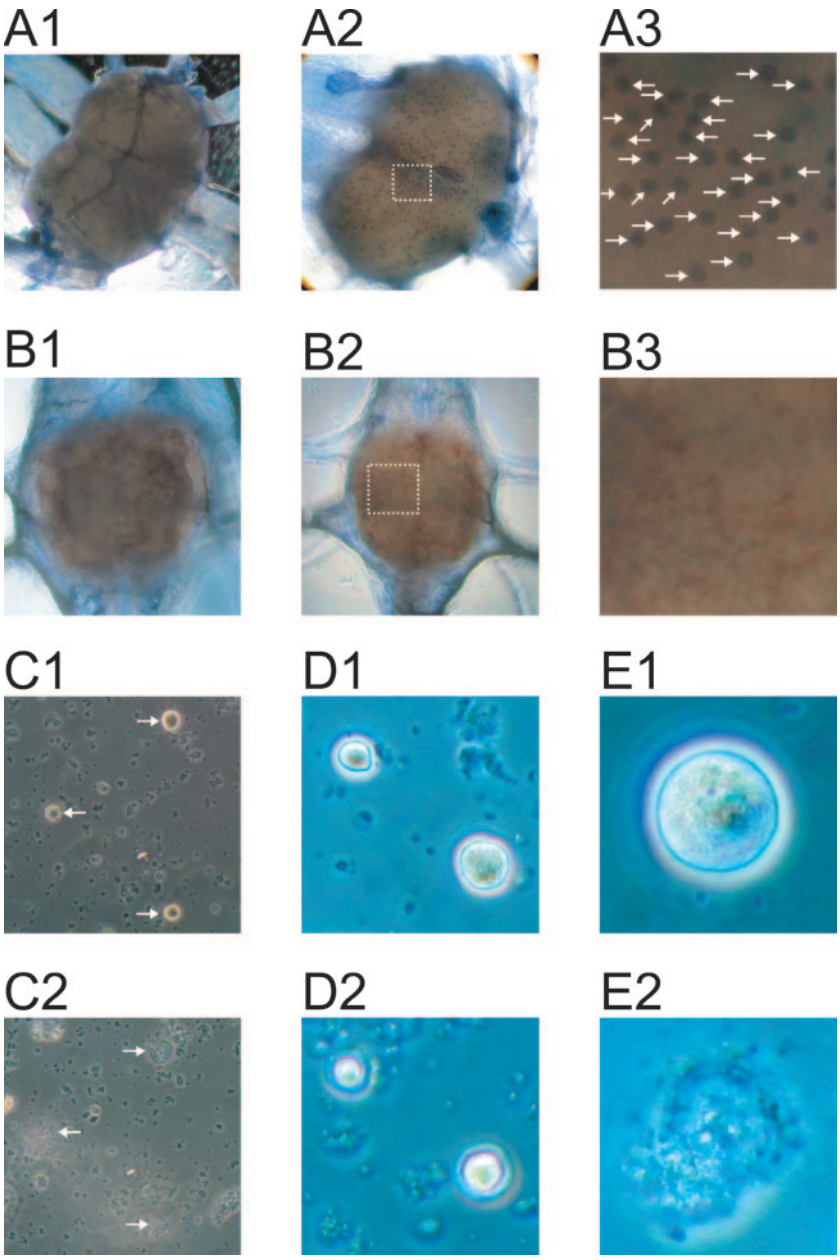

FIG. 6. Effects of sphaericolysin on the nervous systems of insects. (A1 to A3) Damage to the third thoracic ganglia of cockroaches as evaluated by trypan blue staining after no treatment of the solution (A1) and after treatment of the solution with sphaericolysin at 50 ng μl^{-1} for 5 min (A2). A3 is an expansion of the area enclosed within the white dotted line in panel A2. White arrows indicate spots stained with trypan blue. (B1 to B3) Third thoracic ganglia of the common cutworms stained with trypan blue after no treatment of the solution (B1) and after treatment of the solution with sphaericolysin at 50 ng μl^{-1} (B2) for 5 min. Panel B3 is an enlargement of the area enclosed with the white dotted line in panel B2. (C1 and C2) Arrows indicate the cockroach neurons before (C1) and after (C2) treatment with sphaericolysin for 5 min at 50 ng μl^{-1}. Application of the solution without the toxin had no effects on the neurons during the experiment time. (D1 and D2) Cockroach neurons before (D1) and after (D2) treatment with sphaericolysin with the Y159A mutation for 15 min. Application of the heated toxin as well as of the solution without the toxin had no effects on the neurons during the experiment time. (E1 and E2) Cutworm neurons before (E1) and after (E2) treatment with sphaericolysin for 5 min at 50 ng μl^{-1}. Application of the solution without the toxin had no effects on the neurons during the experiment time.

sected from the German cockroaches were barely stained with trypan blue in the absence of the 53-kDa toxin, whereas they displayed various blue spots when treated with the toxin at a concentration of 50 ng μl^{-1} ($n = 4$) (Fig. 6A1 to A3), indicating the strong damage to the neurons. To confirm this, the cockroach neurons dissociated from the third thoracic ganglion were treated with the toxin at a concentration of 50 ng μl^{-1}. The neurons were lysed completely within 5 min after treatment with the toxin ($n = 5$) (Fig. 6C1 and C2). In contrast, neither the heat-treated toxin nor the Y159A mutant had any clear cell lysis activity, even after treatment for 15 min ($n = 3$ and 3, respectively) (Fig. 6D1

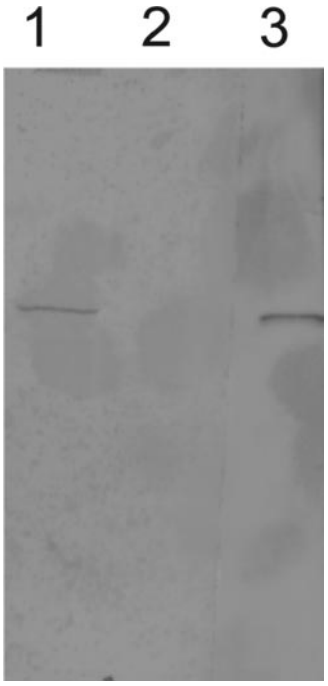

FIG. 7. Detection of sphaericolysin in the carcasses of cutworms injected with A3-2. Lane 1, homogenate of the cutworms injected with A3-2 suspension; lane 2, homogenates of cutworms injected with 0.8% NaCl solution; lane 3, sphaericolysin (250 ng). For lanes 1 and 2, 30 insects were homogenized in 5 ml PBS, 10 μl of which was loaded after dilution with an equal volume of the sample dye.

and D2). The intact ganglia of cutworms were not stained with trypan blue ($n = 3$) (Fig. 6B1 to B3), although the neurons dissociated from the ganglia were rapidly lysed by this toxin ($n = 5$) (Fig. 6E1 and E2).

Immunological detection of sphaericolysin in the carcasses of cutworms injected with *B. sphaericus* A3-2. To examine whether sphaericolysin contributes to the death of common cutworms injected with A3-2, the homogenates of the cutworm carcasses resulting from the injection of the bacteria were subjected to Western analysis using antisera against the toxin. Whereas no toxin band was detected from the cutworms injected with 0.8% NaCl solution (Fig. 7, lane 2), a band was detected at a concentration of about 10 ng μl homogenate^{-1} (in Fig. 7, lane 1 was loaded with 10 μl of the carcass homogenates). From this and the finding that the total volume of the homogenates was greater than the volume of buffer (5,000 μl) used for homogenizing carcasses, the toxin amount produced in a single cutworm was estimated to be greater than 1,600 ng (10 × 5,000/30 larvae), which is greater than the minimum dose (500 ng) to kill a caterpillar.

DISCUSSION

It has been reported that most strains of *B. sphaericus* subgroup IIA exhibit mosquitocidal activity against house mosquitoes (*Culex* spp.) (9, 26, 31) using binary toxin, Mtx1, and Mtx2 (10, 17, 20, 24, 25). In contrast, the isolate A3-2 was unable to kill the mosquitoes, consistent with its lack of mosquitocidal toxin genes (Fig. 1A and B), yet the DNA-DNA hybridization test as well as morphological and physiologic properties indicated that it is a member of *B. sphaericus* subgroup IIA. Nevertheless, the culture broth of the isolate had insecticidal activity when injected into German cockroaches, suggesting the presence of unknown insecticidal factors. Thus, an active principle secreted by A3-2 into the culture broth was purified and characterized. The cloning of the entire gene encoding the protein has led to a finding of an undecapeptide sequence, ECTGLAWEWWR, which is generally seen in the CBCs (1, 4, 8, 32). Consistent with this, sphaericolysin showed hemolytic activity against rat erythrocytes and formed many pores on erythrocyte membranes as revealed by TEM (Fig. 5). It has been reported that the Y181A mutation of perfringolysin O from *Clostridium perfringens* produces a toxin capable of assembling into an oligomeric complex but incapable of inserting its amphipathic β-hairpins into the target membranes (12). To investigate the role of this residue in the insecticidal toxin as well as the hemolytic activities of the sphaericolysin, tyrosine 159 of sphaericolysin, which corresponds to tyrosine 181 of perfringolysin O, was replaced with an alanine. As was the case for perfringolysin O, the Y159A mutation in sphaericolysin led to a marked reduction of its hemolytic activity as well as its insecticidal activity, suggesting a key role of this residue in these two activities. Therefore, the pore-forming activity seems to be implicated in the insecticidal activity. Reduction of the insecticidal activity of sphaericolysin by coinjection with cholesterol suggests an involvement of cholesterol binding in toxicity.

The cockroaches injected with sphaericolysin became paralyzed within a short period. A direct interpretation of this result is that this toxin attacks the system that controls motility. The cockroach thoracic ganglion neurons died rapidly when treated with the toxin regardless of whether they were dissociated or not. Such a cell lysis action on the nervous system seems to result in insect paralysis.

The ganglia dissected from the common cutworms exhibited much lower toxin sensitivity than the cockroach ganglia as evaluated using trypan blue, even though the dissociated cutworm neurons were as sensitive to sphaericolysin as the cockroach neurons. Thus, it is conceivable that the accessibility of toxin to neurons determines the overall toxin sensitivity of the insect nervous system. Taking all of the results into consideration, the insect nervous system is a possible target site of sphaericolysin insecticidal activity. However, the toxin may also attack other tissues, as cholesterol is widely distributed in insects.

We have isolated various insecticidal bacteria against common cutworms from a digestive organ of *M. bore* ant lions (22). Of the insecticidal bacteria, *B. sphaericus* A3-2 was found to produce sphaericolysin in this study. Since ant lions inject their regurgitated fluid into their prey, it was investigated whether toxin is present in the regurgitated fluid. However, no toxin was detected when analyzed using the polyclonal antibodies (data not shown). Nevertheless, the toxin was found to be produced in the caterpillars when A3-2 was injected (Fig. 7). The amount of toxin produced in the cutworms was greater than the minimum lethal dose (500 ng) for the caterpillars. All these results suggest that sphaericolysin might be secreted by

A3-2 after injection, promoting the death of paralyzed prey, rather than directly helping ant lions paralyze prey.

In conclusion, we have purified a novel cytolysin, sphaericolysin, with a molecular mass of 53 kDa from the culture broth of an insecticidal bacterium, A3-2, which belongs to *B. sphaericus* subgroup IIA. The sphaericolysin had a unique N-terminal domain and showed a strong hemolytic activity, which resulted from its pore-forming action via the interactions with cholesterol in the membranes. Also, the sphaericolysin was able to lyse cockroach neurons rapidly, suggesting that the paralysis of insects observed in a short period after injection is, at least in part, due to its attack on the nervous system. The sphaericolysin-encoding gene was also detected in DSM1867, a type strain of *B. sphaericus* subgroup IIA (data not shown), suggesting that other *B. sphaericus* subgroup IIA strains may also possess the toxin gene. It has been reported that insecticidal *B. sphaericus* is, like *Bacillus thuringiensis*, completely safe to other nontarget organisms, such as humans, domesticated animals, and wildlife, and to the environment (11). However, sphaericolysin is active not only on insect neurons but also on hemocytes. Thus, further studies are needed to examine whether commercial *B. sphaericus* subgroup IIA strains produce this toxin.

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