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New Gene from Nine *Bacillus sphaericus* Strains Encoding Highly Conserved 35.8-Kilodalton Mosquitocidal Toxins

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A new gene encoding a 35.8-kDa mosquitocidal toxin (Mtx3; 326 amino acids) was isolated from *Bacillus sphaericus* SSII-1 DNA. Mtx3 is a new type of mosquitocidal toxin with homology to the Mtx2 mosquitocidal toxin of *B. sphaericus* SSII-1, the ε-toxin of *Clostridium perfringens*, and the cytotoxin of *Pseudomonas aeruginosa*. The *mtx3* gene is highly conserved and widely distributed in both high- and low-toxicity mosquito larvicidal strains of *B. sphaericus*.

More than 380 *Bacillus sphaericus* strains which are toxic to the larvae of mosquitoes in at least three genera (*Culex*, *Aedes*, and *Anopheles*) have been identified (1, 5). These strains have been subdivided into the low-toxicity strains (50% lethal concentration [LC_{50}], ~10^5 cells per ml) and the high-toxicity strains (LC_{50}, 10^2 to 10^3 cells per ml) (1, 14). Until recently, only two types of mosquitocidal toxin have been found in *B. sphaericus*: a binary toxin (comprising 51.4- and 41.9-kDa proteins) produced during sporulation in high-toxicity strains (1) and a 100-kDa toxin synthesized in both the low- and high-toxicity strains (18). To date, few mosquitocidal-toxin genes have been isolated and characterized (13). It is important to isolate and identify new and potent mosquitocidal toxins for use as potential biocontrol agents (14). In this paper, we describe the isolation, characterization, and distribution among *B. sphaericus* strains of a new type of mosquitocidal-toxin gene.

Cloning of the *mtx3* gene. Chromosomal DNA was prepared according to a published method (4). *Cla*I-digested chromosomal DNA fragments were separated in a 1% agarose gel, and fragments above 5 kb were isolated from the gel by using the GeneClean kit (Bio 101, Inc.). The isolated fragments were ligated into *Acc*I-digested, alkaline phosphatase-treated plasmid pUC18 (19) and transformed into competent *Escherichia coli* DH5α. Six hundred white recombinant colonies from the library described above were grown overnight at 37°C in 1 ml of Luria-Bertani medium containing 100 μg of ampicillin per ml. The cells were harvested by centrifugation, washed once with water, and fed to five second-instar larvae of *Culex quinquefasciatus*. A toxicity test was performed at 30°C for 24 h (8). A toxic clone, pS57, with an insert of ~7 kb was identified as the parent clone of the new toxin gene. Subclones were produced from pS57 to localize the region within the DNA insert which encodes the protein toxic to *C. quinquefasciatus* larvae. After *Xba*I digestion of pS57, a subclone of 3.1 kb, pSX18, was still toxic to larvae. pSX18 was further digested with *Pst*I and *Kpn*I, and the resulting two fragments of 2.0 and 1.1 kb, respectively, were cloned into *Pst*I- and *Kpn*I-digested pUC18 to give pPK1 and pPK2, respectively. A toxicity assay revealed that only pPK1 (insert, 2.0 kb) was toxic to larvae, and it was, therefore, chosen for sequencing and further analysis.

Expression of Mtx3 fused to glutathione *S*-transferase in *E. coli*. When pPK1 was used as the template, two primers, 5′-GGGGGGTACCCCATGGAAGGAGATAGTAATGTTAAAGAGAATCAAAGT (N1) and 5′-GGGCTGCAGAAAATCGATAAACTGCAGGAGTATGTTTTTTCATCTGTCTAC(N2), were used to PCR amplify the *mtx3* gene sequences coding for amino acids 31 to 326 (i.e., Mtx3 lacking the putative leader sequence; see below). The PCR product was digested with *Nco*I and *Cla*I and ligated into the large (vector) fragment of *Nco*I- and *Cla*I-digested plasmid pTH21 (17) such that codons 31 to 326 of the *mtx3* gene were fused in frame to the 3′ end of the glutathione *S*-transferase (GST) gene (16). The resultant plasmid, pXH1, was verified by DNA sequencing. Methods both for the induction of synthesis of GST fusion proteins in *E. coli* (16) and for toxicity assays for measuring the mosquito larvicidal activity (LC_{50}) of the live recombinant *E. coli* cells have been described previously (17).

Sequence analysis. Computer analyses were performed by using the following software: DNASTAR package (DNASTAR, Inc.), PC/GENE package (IntelliGenetics), and GCG package (University of Wisconsin).

Southern blot analysis. Total DNA was prepared from 14 strains of *B. sphaericus* (4). Approximately 5 μg of each DNA preparation was completely digested with *Cla*I and separated by electrophoresis in a 1% agarose gel. The DNA was blotted onto Hybond-N+ membranes (Amersham International). The 2.0-kb *Pst*I-*Kpn*I fragment of pPK1 was labelled by using the enhanced chemiluminescence labelling kit (Amersham International) and was used as a probe. The blot was developed as described by the manufacturer (Amersham International).

Cloning of *mtx3* genes from strains other than SSII-1. The primers 5′-GGGGGGTACCCCATGGAAATGAAAAACAAAGCAAAAGTAATATTAATGGGA (J1) and 5′-GGGCTGCAGAAAATCGATAAAGAGTATGTTTTTTCATCTGTCTACGAA (X3) were used to perform PCR amplification of the genomic DNAs of different strains of *B. sphaericus* to confirm the existence of the toxin gene and to perform nucleotide sequence analysis on two independent clones from each strain to exclude PCR errors.

Features of the open reading frame and transcriptional and translational control elements. The DNA sequence of the

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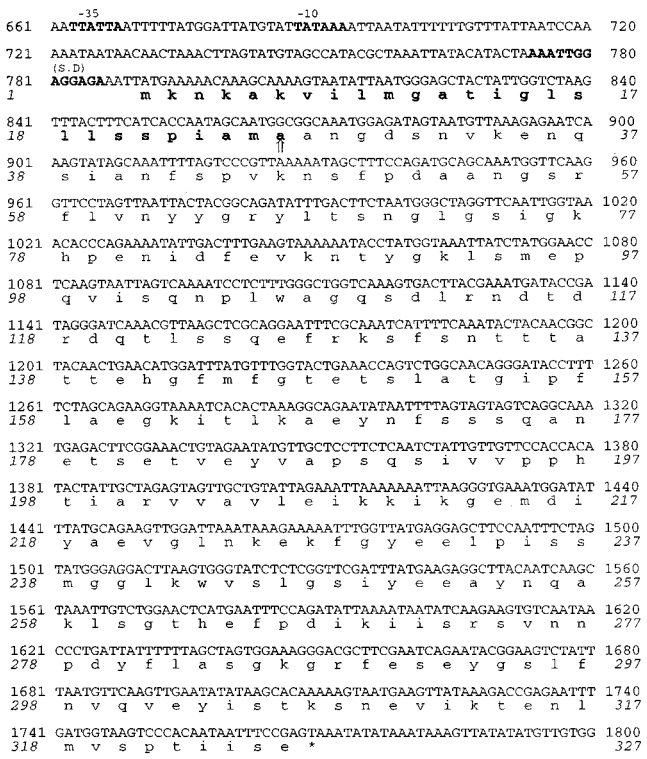

FIG. 1. Nucleotide sequence of the DNA fragment encoding the 35.8-kDa toxin of *B. sphaericus* SSII-1. The deduced amino acid sequence is shown below the nucleotide sequence. The possible Shine-Dalgarno (S.D) region and −35 and −10 consensus sequences controlling translation and transcription of the *mtx3* gene, respectively, are in boldface type. The putative N-terminal signal peptide is in boldface type, and the vertical arrow indicates the possible cleavage site of a signal peptidase.

2.0-kb *Kpn*I-*Pst*I fragment from plasmid pPK1 contains a single long open reading frame commencing at nucleotide (nt) 791 and terminating at nt 1768 (Fig. 1). A putative ribosome binding site for the mRNA transcribed from the *mtx3* gene (AAAUUGGAGGAGA, nt 774 to 786) (Fig. 1) is located 5 nt upstream from the initiation codon AUG and shows a 9-base homology to the 3′ terminus of *Bacillus subtilis* 16S rRNA, UCUUUCCUCCACUAG (11). Similar ribosome binding sites have also been found upstream of other *B. sphaericus* genes, for example, AAAAAGAGGUG in the 100-kDa-toxin gene of strain SSII-1 (18) and AUAUUGGAGAG in the 31.8-kDa-toxin gene of strain SSII-1 (19). The putative promoter sequences of the *mtx3* gene, TATAAA (nt 688 to 693) and TTATTA (nt 663 to 668) (Fig. 1), show 100 and 50% homology, respectively, to the consensus sequences for promoters recognized by the σ^{55} RNA polymerase during the vegetative phase of *B. subtilis* growth (TATAAA at nt −10 and TTGACA at nt −35 from the transcription start site) (11). This is consistent with the observation that the synthesis of mosquito larvicidal activity in the low-toxicity strain *B. sphaericus* SSII-1 occurs during vegetative growth (12).

Features of the toxin and its expression in *E. coli*. The open reading frame encodes a protein (Mtx3) containing 326 amino acids with a calculated molecular mass of 35.8 kDa (Fig. 1). The synthesis of a fusion protein comprising GST fused to Mtx3 lacking the putative N-terminal leader sequence was induced in *E. coli*. The recombinant *E. coli* was moderately toxic to *C. quinquefasciatus* larvae (LC_{50}, 9.55×10^5 cells per ml) and weakly toxic to *Aedes aegypti* larvae (LC_{50}, 1.95×10^7 cells per ml). Control *E. coli* in which GST-Mtx3 was not induced was nontoxic to the larvae of both species of mosquitoes (LC_{50}, $\geq 10^8$ cells per ml). These results demonstrate that the long open reading frame codes for a mosquitocidal toxin.

Significant homology was found between the 35.8-kDa Mtx3 protein and the 31.8-kDa mosquitocidal toxin (Mtx2) of *B. sphaericus* SSII-1 (19), the 33-kDa ε-toxin of *Clostridium perfringens* (7), and the 31.68-kDa cytotoxin of *Pseudomonas aeruginosa* (6), particularly in the middle region of Mtx3 (Fig. 2). Overall, the 35.8-kDa protein from *B. sphaericus* exhibits 38% identity to the 31.8-kDa Mtx2 toxin of *B. sphaericus* SSII-1 over a stretch of 135 amino acids, 30% identity to the ε-toxin of *C. perfringens* over a stretch of 144 amino acids, and 27% identity to the cytotoxin of *P. aeruginosa* over a stretch of 63 amino acids (Fig. 2).

The N terminus of the deduced Mtx3 protein has features characteristic of signal peptides of gram-positive bacteria (Fig. 1) (3). Within the putative signal peptide of 26 amino acids, the sequence NKAKVILMGATIGLSLLSSPIAMA is predicted to be a potential transmembrane helix. As toxicity of *B. sphaericus* SSII-1 is associated with the insoluble pellet fraction, it is possible that in the intact cell this leader sequence is not cleaved off and it functions as a transmembrane domain to attach Mtx3 to membranes. Only later following cell lysis might the leader be cleaved off. Insecticidal toxins of bacteria are

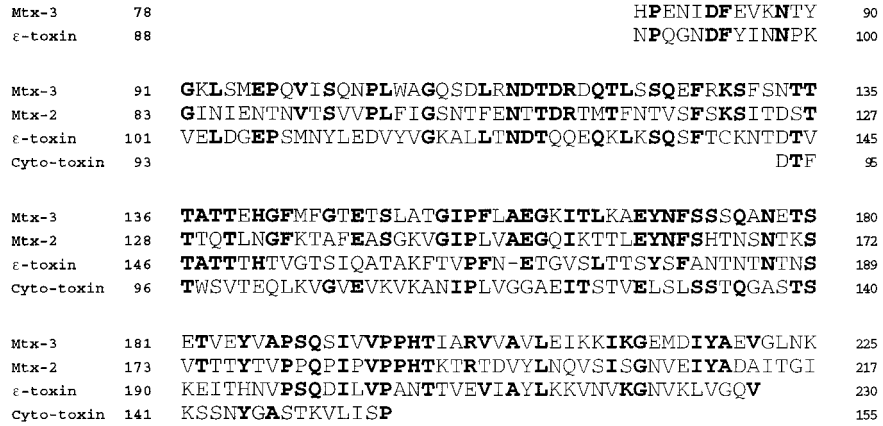

FIG. 2. Sequence alignments of part of the 35.8-kDa Mtx3 protein with the 31.8-kDa Mtx2 toxin of *B. sphaericus* SSII-1 (19), the ε-toxin of *C. perfringens* (7), and the cytotoxin of *P. aeruginosa* (6). Amino acids which are identical in Mtx3 and at least one other toxin are in boldface type.

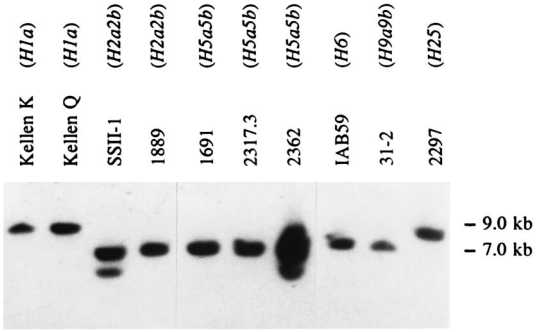

FIG. 3. Southern blot hybridization of *Cla*I-digested DNA from various *B. sphaericus* strains to an *mtx3* toxin gene probe. The sizes of the DNA markers are shown at the right. The H serotype (5) is shown above the strain name. Strains 1691, 2317.3, 2362, IAB59, and 2297 are high-toxicity strains, whereas Kellen K, Kellen Q, SSII-1, 1889, and 31-2 are low-toxicity strains.

typically synthesized as protoxins which are proteolytically activated in the insect gut (1, 14). The ε-toxin of *C. perfringens* has an N-terminal leader of 14 amino acids which is cleaved by proteases, resulting in activation of the toxin (2), but it remains to be determined whether the related 35.8-kDa protein is also a protoxin requiring activation by removal of the N-terminal leader.

The precise mechanisms of action of the *Clostridium* ε-toxin and *Pseudomonas* cytotoxin are unknown, but these toxins are believed to form pores on susceptible target cells leading to an inflow of water, a loss of osmotic balance, and, consequently, cell lysis (9, 10, 15). As Mtx3 appears related to these toxins, it is possible that Mtx3 is also a pore former in common with many other insecticidal toxins.

Distribution and sequence of the *mtx3* gene in *B. sphaericus* strains. Southern blot hybridization of the *mtx3* gene to *Cla*I-digested, gel-fractionated DNA from different *B. sphaericus* strains showed that the 35.8-kDa gene is widely distributed in both high- and low-toxicity strains of *B. sphaericus* (Fig. 3). In serotypes H1a and H25, the size of the hybridizing band is about 8.5 to 9 kb, whereas in the other strains examined (H2a2b, H5a5b, H6, and H9a9b), the size of the major band is about 7 kb. To confirm the existence of the *mtx3* gene in *B. sphaericus* strains other than SSII-1, PCR amplification of DNA from strains 2297, 1593, 2362, 1691, 2317.3, Kellen Q, IAB59, and 31-2 was performed with oligonucleotide primers J1 and X3 (see above). The derived DNA sequences of *mtx3* from SSII-1 and 31-2 were identical. The predicted amino acid sequences of Mtx3 from SSII-1 (Fig. 1), 1593, 2362, 1691, 2317.3, and 31-2 were also identical, but Mtx3 from 2297, Kellen Q, and IAB59 had a single conservative amino acid change (Val-45 to Ile) compared with Mtx3 from SSII-1. Thus, the *mtx3* gene is highly conserved among both high- and low-toxicity strains of *B. sphaericus*. The conservation of the amino acid sequence of Mtx3 in *B. sphaericus* strains collected from diverse regions of the world suggests that this protein plays an important role in the ecological niche of the bacterium.

Nucleotide sequence accession numbers. The GenBank accession numbers for the gene sequences reported in this article are U42328, U42329, U42330, U42331, U42332, U42333, U42334, and U42335.

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