Cloning and Characterization of a Cytolytic and Mosquitocidal δ -Endotoxin from *Bacillus thuringiensis* subsp. *jegathesan*

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A cytolytic toxin gene encoding a 30.1-kDa Cyt2Bb1 toxin protein from *B. thuringiensis* subsp. *jegathesan* was cloned employing a limited-growth PCR screening method with forward and reverse oligonucleotide primers designed from N-terminal amino acid sequences of native and trypsin-cleaved protein, respectively. The expressed protein showed little cross-reactivity to the antibody raised against the Cyt1Aa protein. Unlike Cyt1Aa and Cyt2Aa expression, there was little or no visible crystal inclusion formation under microscopic observation. The amino acid sequence alignment indicated 31 and 66% identity to Cyt1Aa and Cyt2Aa, respectively. The sequence alignment for five known cytolytic proteins indicated three highly conserved regions, two in the loop regions between α -helices and β -sheets and one in the loop region between β -sheets 5 and 6. β -Blocks 4 to 7 are also conserved, not only structurally but also among the amino acids in the hydrophobic faces. Mosquitocidal activity assays indicated that the Cyt2Bb toxin had less toxicity than Cyt1Aa and had about 600-times-lower toxicity than the wild-type whole toxin crystal. However, both the Cyt2Bb and the Cyt1Aa toxin showed comparable levels of hemolytic activity.

Bacillus thuringiensis, a gram-positive endospore-forming bacterium, produces an insecticidal parasporal crystal inclusion(s) outside the exosporium but within the sporangium. Many strains of this species are insecticidal to lepidopteran, coleopteran, and dipteran insects, many of which are important in agriculture and in animal and human health. Due to the potential use of *B. thuringiensis* in insect control, a number of isolates have been identified and the insecticidal δ -endotoxin genes and proteins have been characterized (16). These δ -endotoxin genes encode a family of Cry (Crystal) proteins. Presently 20 Cry protein classes, based on their deduced amino acid sequence homology, are known (http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html).

Cytolytic toxins that are structurally unrelated to the Cry protein family are also produced by some *B. thuringiensis* strains, and these are classified as Cyt (cytolytic) proteins. The Cyt toxins are distinct from the Cry toxins in a number of ways. First, the Cyt toxins have to date been reported only in dipteran-specific *B. thuringiensis* strains, whereas the Cry toxins are found in *B. thuringiensis* strains with wide host selectivity (11). Second, only one Cyt toxin is present in a given *B. thuringiensis* strain, whereas two or more subclasses of Cry toxins can often be found in a strain of *B. thuringiensis* (11). Third, only the Cyt toxins cause cytolysis of a variety of eukaryotic cells and erythrocytes, even though both the activated Cyt toxins and the Cry toxins can form pores in lipid bilayers (19, 31, 34).

Two of the Cyt toxins, Cyt1Aa1 from *B. thuringiensis* subsp. *israelensis* and Cyt2Aa1 from *B. thuringiensis* subsp. *kyushuensis*, have been cloned and characterized (23, 35). The presence of Cyt toxins also has been reported for a variety of other mosquitocidal *B. thuringiensis* strains, including *B. thuringiensis* subsp. *fukuokaensis* (40), subsp. *jegathesan* (18), subsp. *darmstadiensis* (9), and subsp. *medellin* (27). The nature of the cytolytic protein in *B. thuringiensis* subsp. *fukuokaensis* is not

known, while the cytolytic protein from *B. thuringiensis* subsp. *darmstadiensis* shares immunological cross-reactivity with Cyt2Aa from *B. thuringiensis* subsp. *kyushuensis* (21). The cytolytic protein from *B. thuringiensis* subsp. *medellin* cross-reacts with anti-Cyt1Aa antibodies (27), and the cloned toxin from this species has high amino acid identity to the Cyt1Aa toxin and is consequently classified as Cyt1Ab1 (http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html). The presence of other Cyt1A-like genes and/or proteins has been investigated by employing both immunological cross-reactivity with the antibody raised against the purified Cyt1Aa protein and Southern analysis with the *cyt1Aa* gene as a probe. A number of mosquitocidal strains of *B. thuringiensis*, including *B. thuringiensis* subsp. *canadiensis*, subsp. *thompsoni*, and subsp. *malaysiensis*, have been shown to carry the Cyt1Aa-like gene and protein (29).

The three-dimensional structure of one of the cytolytic toxins, Cyt2Aa, has been elucidated, and it consists of a single domain with two outer layers of α -helix hairpins wrapped around mixed β -sheets that are likely to form a transmembrane β -barrel (25). Sequence alignment of the Cyt1Aa and Cyt2Aa proteins also showed that this α/β architecture is presumably highly conserved among different Cyt toxins (25).

The cytolytic toxin, when solubilized in the alkaline mosquito midgut, is released as a dimeric protoxin. Upon proteolytic activation of the protoxin at both the N and C termini (1, 3, 14), a monomeric toxin is obtained that acts on the midgut epithelial cell membrane (25). The cytolytic activity is believed to be mediated by the ability of the activated toxin to bind unsaturated phospholipids in the cell membrane (14, 33), forming toxin aggregates on the cell membrane (6, 26). These toxin aggregates then form cation-specific pores (19) that cause osmotic imbalance resulting in cell lysis and insect death (11, 20). The Cyt toxins act on a variety of cultured cell lines and mammalian erythrocytes, causing cytolysis probably by a similar mechanism (12, 14, 32).

The cytolytic toxins are also mosquitocidal, although their mosquitocidal activity is generally lower than that of the mosquitocidal Cry toxins (5, 7). However, the Cyt toxins act synergistically with mosquitocidal Cry toxins and play a crucial

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role in the overall activity of the intact parasporal inclusions (5, 7, 28, 39). Furthermore, the Cyt toxins also play an important role in overcoming mosquito larvae resistance to the Cry11A toxin (10a).

Previously we reported the characterization of *B. thuringiensis* subsp. *jegathesan*, which has high mosquitocidal activity, and showed the presence of a cytolytic toxin by hemolysis assay of the activated whole toxin (18). Here we describe the cloning, expression, and mosquitocidal and cytolytic activities of this toxin from *B. thuringiensis* subsp. *jegathesan*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. thuringiensis* subsp. *jegathesan*, obtained from the Institute for Medical Research, Kuala Lumpur, Malaysia, has been previously characterized (18). For transformation and expression of the cytolytic toxin gene, the cry mutant strain of *B. thuringiensis* subsp. *kurstaki* was used. All the *B. thuringiensis* strains were maintained on nutrient agar (Difco Inc.). *Escherichia coli* XL-2 ultracompetent cells (Stratagene) were used for the initial cloning of the toxin gene. Both for cloning and expression of the cytolytic toxin gene, the *E. coli*-*B. thuringiensis* shuttle vector pHT315 (2) was used. Selection was made with 25 µg of erythromycin ml⁻¹ for *B. thuringiensis* transformants and 50 µg of ampicillin ml⁻¹ and 12.5 µg of tetracycline ml⁻¹ for *E. coli* transformants. For subcloning and sequencing of the gene, the plasmid pBluescript SK(+) (Stratagene) was used.

Protein analysis. For preparation of the whole toxin crystal from *B. thuringiensis* subsp. *jegathesan*, the wild-type cell was streaked on nutrient agar plates and incubated at 30°C for 3 days to induce sporulation and crystal formation. The spore-crystal mixture was harvested and washed three times in 1 M NaCl-25 mM EDTA and then resuspended in water. The mixture was then loaded onto a 38 to 56% NaBr step gradient and centrifuged at 20,000 rpm at 4°C for 2 h in a Beckman SW28 rotor. The toxin crystal layer was harvested and washed three times with 25 mM EDTA to remove NaBr.

For toxin solubilization the purified crystals were washed briefly in a sodium carbonate buffer of pH 9.0, pH 10.5, or pH 11.5, and then resuspended in the same buffer. The toxin was solubilized with stirring for 1 to 4 h in the presence of 1 mM dithiothreitol (DTT). Most of the 28-kDa protein was preferentially solubilized in 50 mM sodium carbonate buffer (pH 10.5). Toxin solubilization was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed with a minigel apparatus (Bio-Rad). The gels were stained with 0.4% Coomassie blue R250. The protein masses were determined by comparison with wide-range molecular weight standards (Sigma Chemical Co.).

The solubilized proteins (10 µg) were digested with either 1 µg of trypsin or chymotrypsin in sodium carbonate buffer for 1 h at room temperature with occasional shaking. The undigested 26-kDa soluble protein and the trypsin-digested 24-kDa protein were separated by SDS-PAGE and then transferred to Immobilon-P membranes (Millipore Corp.) in 10 mM 3-cyclohexyl-amino-1-propanesulfonic acid buffer with a Bio-Rad minigel transfer unit. N-terminal amino acid sequencing was then performed for both the 24- and 26-kDa proteins at the Biotechnology Instrumentation Facility, University of California, Riverside, Calif.

For Western blot analysis, the proteins were transferred from SDS-PAGE gels onto Immobilon-P nitrocellulose membranes and then probed with rabbit antibody developed against the purified 24-kDa Cyt1Aa toxin of *B. thuringiensis* subsp. *israelensis* by methods described previously (13). Goat anti-rabbit immunoglobulin G-alkaline phosphatase was used as the secondary antibody, and chromogenic development was then achieved with nitroblue tetrazolium chloride-5-bromo-4-chloro-3-indolyl phosphate tablets (0.3 and 0.15 mg ml of water⁻¹, respectively) (Sigma Chemical Co.).

Protein concentrations were measured with a Bradford method-based bicinchoninic acid protein assay kit following the manufacturer's instructions (Pierce Chemical Co.) with bovine serum albumin as the standard.

Cloning and sequencing of the toxin gene. In order to clone the cytolytic toxin gene, two degenerate oligonucleotides were designed. A forward and a reverse primer were designed based on the amino acid sequences of the undigested and the trypsin-digested proteins, respectively. These primers were used for PCR with megaplasmid DNA prepared from *B. thuringiensis* subsp. *jegathesan*. The primers were then used to screen a plasmid library made with *Xba*I-digested DNA cloned into the *E. coli*-*B. thuringiensis* shuttle vector pHT315. Isolation of the toxin gene from the library was performed by limited-growth PCR screening (30).

Briefly, *Xba*I-digested megaplasmid DNA was ligated into shuttle vector pHT315 and transformed into *E. coli* XL-2 ultracompetent cells to obtain the plasmid library. Megaplasmid DNA was isolated from *B. thuringiensis* subsp. *jegathesan* as previously described (18). The entire library was divided into 100 aliquots, and each aliquot was placed in 2 ml of Luria-Bertani broth containing 50 µg of ampicillin ml⁻¹ and grown for 3 h to allow 2 to 3 cell doublings. One-milliliter aliquots from each of the 100 tubes were taken and stored at 4°C.

FIG. 1. SDS-13% PAGE analysis and Coomassie blue-stained gel of the solubilized and trypsin-digested protein. Lane 1, 5 µg of total protein of parasporal inclusion from *B. thuringiensis* subsp. *jegathesan*; lane 2, 5 µg of total protein solubilized in sodium carbonate buffer, pH 10.5; lane 3, the same amount of protein digested with trypsin. At pH 10.5, the 26-kDa protein is solubilized without significant contamination with neighboring protein bands. The 26-kDa unprocessed (lane 2) and the 24-kDa trypsin-digested protein (lane 3) were subjected to N-terminal sequencing that facilitated the design of oligonucleotide primers for PCR screening and cloning of the *cyt2Bb* gene. Molecular mass is indicated in kilodaltons.

The remaining 1-ml aliquot was grown overnight. Then 100 µl from the overnight cultures was pooled into 10 groups of 10 aliquots, and DNA from these pooled cultures was extracted. PCR was then performed with this DNA as the template and with the forward and reverse primers. Positive pools were identified, and then DNA from individual tubes within the pool was analyzed. All the bacteria in the positive tubes were then subjected to additional rounds of limited-growth screening. After three rounds of screening the bacterial culture was transferred to 10 Luria-Bertani broth-ampicillin plates, and each individual colony from the plate was subjected to PCR to isolate the positive clone.

Nucleotide sequences were determined by the dideoxy chain termination method with Sequenase 2.0 (Amersham) and α-³²S-dATP (Amersham) following the manufacturer's instructions.

Mosquitocidal and hemolytic activity. Mosquitocidal activity was determined in 5 ml of water with 10 early-second-instar larvae of *Aedes aegypti*. For the bioassay, the spore-crystal mixture was used without NaBr gradient purification. All assays were performed in triplicate. The mortality was scored after 36 h. The acryalliferous strain of *B. thuringiensis* subsp. *kurstaki* and water only were both used as negative controls.

Hemolytic activity was assessed with rabbit blood. Blood was collected and diluted 1:4 in phosphate-buffered saline (PBS) buffer, pH 7.5. The diluted rabbit blood cells were washed two times in the same buffer, and a 75-µl aliquot was diluted in 225 µl of PBS and then incubated with the various concentrations of the activated toxin. After incubation at 37°C for 30 min, the mixture was centrifuged at 4,000 rpm for 4 min, and the supernatant was analyzed for hemoglobin release by determining the absorption at 570 nm. The percent lysis values were obtained by comparison to 100% lysis, obtained by incubating the blood with 225 µl of water, or to 0% lysis, obtained with PBS buffer.

The toxins used for hemolysis were prepared as follows. The cytolytic toxin from *B. thuringiensis* subsp. *morrisoni*, Cyt1Aa, was activated with trypsin, while the cytolytic toxin from *B. thuringiensis* subsp. *jegathesan*, Cyt2Bb, was activated with trypsin and chymotrypsin. Each spore-toxin mixture of Cyt1Aa- and Cyt2Bb-expressing cells was lysed with glass beads in 50 mM sodium carbonate buffer, pH 10.5, for 5 min and incubated for 2 h in the presence of 1 mM DTT with stirring to solubilize the toxin. The mixture was centrifuged, and the supernatant was collected and then incubated with trypsin or a trypsin-chymotrypsin mixture at 37°C for 1 h.

RESULTS

Cloning of the cyt gene. In order to clone the cytolytic toxin gene, the intact toxin crystals from *B. thuringiensis* subsp. *jegathesan* were purified and solubilized in alkaline buffer with various pHs and incubation times in the absence or presence of DTT (data not shown). With incubation in 50 mM sodium carbonate buffer, pH 10.5, in the absence of 1 mM DTT for one h, most of the 26-kDa protein was preferentially solubilized together with proteins of 72 and 58 kDa and two minor proteins of around 37 and 39 kDa (Fig. 1, lane 2). The solu-

FIG. 2. Restriction map and nucleotide sequence of *cyt2Bb*. (A) Restriction map of the 1.7-kb *XbaI-PstI* fragment cloned in pHT315 containing the *cyt2Bb* gene. The relative size and direction of the gene are indicated by an arrow. B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; M, *Mun*I; Nh, *Nhe*I; Nr, *Nru*I; P, *Pst*I; S, *Sca*I; X, *Xba*I. (B) Complete sequence of 1,719 nucleotides containing the *cyt2Bb* ORF. The amino acid sequence of the Cyt2Bb protein, corresponding to the genetic sequence from nucleotides 416 to 1207, predicts a 30.1-kDa protein. A putative ribosomal binding site is underlined and indicated as RBS. Putative early -35 and -10 promoters are also underlined. Two putative 3' stem-and-loop structures are indicated with arrows. The amino acid sequences that were determined by N-terminal sequencing of both the native and trypsin-processed proteins are underlined. The accession number for the Cyt2Bb toxin is U82519.

bilized protein was subjected to both trypsin and chymotrypsin digestion. The 26-kDa protein band was reduced in size to around 24 kDa after trypsin digestion (Fig. 1, lane 3) and to slightly less than 24 kDa after chymotrypsin digestion (data not shown for chymotrypsin). Both the 26-kDa and the trypsin-digested 24-kDa proteins were subjected to N-terminal sequencing. A 21-amino-acid sequence for the 26-kDa protein was determined unambiguously as MYTKLNSLEINED YQYSRPI, and an 11-amino-acid sequence for the trypsin-digested protein was identified as HITLTPSSDI.

Three degenerate oligonucleotide primers were designed based on the two amino acid sequences. Two forward primers, AAATGTA(TC)ACIAA(AG)AA(TC)(TC)T and CTIGA(AG)AT(ATC)AA(TC)GA(AG)GA(TC)TA, based on the amino acid sequences MYTKLN and LEINEDY, respectively, were made. A reverse primer, (ATG)AT(AG)TC(AG)CTIG AIGGIACIGT, corresponding to the amino acid sequence

TVPSSDI, was also synthesized. These primers were initially used in PCRs to identify a 114-bp fragment by using megaplas-mid DNA from *B. thuringiensis* subsp. *jegathesan*. Subsequently these primers were used to isolate the cytolytic toxin-contain-ing clone from a *XbaI*-digested library of megaplas-mid DNA in the shuttle vector pHT315 by employing a limited-growth PCR screening method previously developed in our laboratory (30). The isolated plasmid clone had a 3.5-kb *XbaI* insert. Further restriction analysis indicated that the cytolytic toxin gene was located in a 1.7-kb *XbaI-PstI* fragment (Fig. 2A), which was subcloned into the pBluescript SK(+) vector to facilitate nu-cleotide sequencing.

The nucleotide sequence was determined in both directions, and an open reading frame (ORF) of 789 bp was identified (Fig. 2B). The ORF encodes for a polypeptide of 263 amino acid residues with a predicted molecular mass of 30,057 Da (Fig. 2B). The predicted amino acid sequences matched the

FIG. 3. SDS-14% PAGE analysis of the expressed Cyt2Bb toxin and immunoblot with antibody against Cyt1Aa. (A) Total spore-toxin mixture (20 μ g) was loaded in SDS-14% polyacrylamide gel and stained with Coomassie blue R250. Lane 1, Cyt2Bb expressed in the acrySTALLIFEROUS strain of *B. thuringiensis* subsp. *kurstaki*; lane 2, *B. thuringiensis* subsp. *jegathesan* wild type; lane 3, acrySTALLIFEROUS strain of *B. thuringiensis* subsp. *kurstaki*; lane 4, Cyt1Aa expressed in the acrySTALLIFEROUS strain of *B. thuringiensis* subsp. *kurstaki*. Several high-molecular-mass bands at about 66 kDa and two bands at about 36 kDa are products of the spore extract of the CryB⁺ strain of *B. thuringiensis* subsp. *kurstaki*. (B) Immunological cross-reactivity analysis with antibody raised against Cyt1Aa. Twenty micrograms of Cyt2Bb and 30 μ g of Cyt1Aa protein expressed in the acrySTALLIFEROUS strain of *B. thuringiensis* subsp. *kurstaki* were loaded in SDS-14% polyacrylamide gel together with the molecular mass standard and transferred onto Immobilon-P membrane. One of the membranes was Coomassie blue stained and is shown in panel a. The other membrane was screened with 1:5,000-diluted antibody against Cyt1Aa and is shown in panel b. Lanes 1, Cyt2Bb; lanes 2, Cyt1Aa. Molecular mass is indicated in kilodaltons.

N-terminal sequences of the undigested and the trypsin-digested proteins.

A Shine-Dalgarno sequence (GGAGG) 8 bp upstream of the ATG start codon, as observed in the *cyt2Aa* gene (23), was observed (Fig. 2B). Analysis of the 5' upstream sequence revealed putative promoter sequences that are highly homologous to those of the *cyt1Aa* gene of *B. thuringiensis* subsp. *israelensis* (36) and the *BtI* promoter of *B. thuringiensis* subsp.

kurstaki (4). The -10 region had 7 of 8 and the -35 region showed 6 of 8 matching sequences (Fig. 2B). The 3' end of the ORF revealed the presence of inverted repeat elements (Fig. 2B).

Expression of the cytolytic toxin protein. As shown above, the 1.7-kb *XbaI-PstI* fragment contained all the regulatory sequences necessary for protein expression. Hence to express the cytolytic toxin protein, the 1.8-kb *PstI* fragment was deleted from the 3.5-kb *XbaI* insert in pH315 and the resulting fragment was self-ligated and then transformed back into *E. coli*. The purified plasmid was then electroporated into the *B. thuringiensis* subsp. *kurstaki* cry mutant. Transformants were screened for the expression of a protein of the expected size and analyzed in SDS-14% polyacrylamide gel. Fig. 3A shows the expressed 26-kDa protein (Fig. 3A, lane 1). Microscopic observation of the *B. thuringiensis* subsp. *kurstaki* cry mutant cells transformed with the cloned *cyt2Bb* gene indicated no visible crystal formation in these strains, and hence it was not possible to purify the cytolytic toxin with NaBr gradients.

Anti-Cyt1Aa polyclonal antibodies barely detected the expressed cytolytic toxin (Fig. 3B, panel b, lane 1), while these antibodies readily detected the Cyt1Aa protein (Fig. 3B, panel b, lane 2). As a negative control, no cross-reactivity was observed with the protein molecular mass markers (Fig. 3B, panel b, lane S).

Sequence homology. The Cyt2Bb sequence was compared with known sequences of other cytolytic toxins with the MegAlign computer program (DNASTAR). The cloned Cyt2Bb1 toxin showed 66 and 31% sequence identity to the Cyt2Aa1 and Cyt1Aa1 proteins, respectively. The sequences for three other cytolytic toxins, Cyt1Ab1, Cyt1Ba1, and Cyt2Ba1, are also available in the GenBank database, version 99.0. The six complete cytolytic toxin sequences were aligned by using the MegAlign program (Fig. 4). Shaded residues in-

FIG. 4. Sequence alignment of the cytolytic toxins from *B. thuringiensis*. The toxin sequences and their accession numbers are as follows: Cyt1Aa1, from *B. thuringiensis* subsp. *israelensis*, X03182; Cyt1Ab1, from *B. thuringiensis* subsp. *medellin*, X98793; Cyt1Ba1, from *B. thuringiensis* subsp. *neoleoensis*, U37196; Cyt2Aa1, from *B. thuringiensis* subsp. *kyushuensis* Z14147; Cyt2Ba1, from *B. thuringiensis* subsp. *israelensis*, U52043; and Cyt2Bb1, from *B. thuringiensis* subsp. *jegathesan*, U82519. Completely conserved amino acids of all six Cyt toxins are shaded, and highly conserved substitutions are shown in boxes. Similar substitution residue groups were accepted as follows: D and E; Q and N; K, H, and R; A, G, I, L, and V; F, W, and Y; and S and T. The corresponding α -helices and β -sheets of the Cyt2Aa toxin (25) are shown below the aligned sequences.

TABLE 1. Mosquitocidal activity of a spore-toxin mixture of Cyt1Aa and Cyt2Bb expressed in a *cryB* mutant strain of *B. thuringiensis* subsp. *kurstaki* tested against early-second-instar larvae of *A. aegypti*

Strain	Toxin composition	LC ₅₀ (μg/ml) ^a
<i>B. thuringiensis</i> subsp. <i>jegathesan</i>	Whole toxin	0.021 (0.011–0.036)
<i>cryB</i> mutant (pCG17)	Cyt1Aa	1.2 (1.0–1.6)
<i>cryB</i> mutant (pCytD.1)	Cyt2Bb	6.8 (5.3–8.4)
<i>cryB</i> mutant		ND ^b

^a LC₅₀s are the average values of three tests. Values in parentheses indicate the range of LC₅₀s in each test.

^b ND, no toxicity detected at 50 μg of total protein ml⁻¹.

dicating sequences that are identical in all six toxins, while boxed sequences represent conserved amino acid substitutions.

Mosquito larvicidal activity. Mosquitocidal activity was determined with early-second-instar *A. aegypti* mosquito larvae. Toxin-spore preparations obtained from cell lysis were used for all strains in the bioassays. The Cyt1Aa toxin, produced with a previously constructed strain in our laboratory (CG17), showed higher mosquitocidal activity than the Cyt2Bb toxin (50% lethal concentrations [LC₅₀s] of 1.2 and 6.8 μg ml⁻¹, respectively) (Table 1). In contrast, the wild-type whole toxin-spore preparation from *B. thuringiensis* subsp. *jegathesan* had an LC₅₀ of 21 ng ml⁻¹ (Table 1). As a negative control, the sporulated acrySTALLIFEROUS *cry* mutant strain of *B. thuringiensis* subsp. *kurstaki* (*cryB* mutant strain), which was used for expression of the Cyt1Aa and Cyt2Bb proteins, showed no toxicity at more than 50 μg of protein ml⁻¹ (Table 1).

Hemolytic assay. Under conditions described in Materials and Methods, the activated Cyt1Aa toxin caused 50% lysis of the rabbit erythrocytes at about 0.9 μg of toxin ml⁻¹. The activated Cyt2Bb showed an almost comparable response, with 50% hemolysis at about 1.3 μg ml⁻¹. The two cytolytic toxins yielded similar dose response curves (Fig. 5).

FIG. 5. Hemolytic activity of the expressed Cyt2Bb and Cyt1Aa toxins. The percent lysis of rabbit erythrocytes was compared to 100% lysis, obtained by incubating the blood with water, or to 0% lysis, obtained with cells incubated in PBS buffer. The data points are mean values of two assays performed in duplicate.

DISCUSSION

We report here the cloning, expression, and the mosquitocidal and cytolytic activities of a toxin from a highly mosquitocidal strain, *B. thuringiensis* subsp. *jegathesan*. Selective solubilization of the cytolytic toxin in 50 mM sodium carbonate (pH 10.5) buffer followed by trypsin digestion facilitated the determination of the N termini of the native and proteolytically cleaved proteins. PCR of megaplasmid DNA with primers designed by using these N termini permitted the isolation of a 114-bp DNA fragment, and sequencing this fragment confirmed the presence of a Cyt2Aa-like toxin. Use of these primers in a subsequent screen of a plasmid library constructed in the *B. thuringiensis*-*E. coli* shuttle vector pHT315 resulted in the isolation of the *cyt2Bb* gene. The *cyt2Bb* gene was detected in a 1.7-kb *Xba*I-*Pst*I fragment and encodes for a 30.1-kDa protein, which in SDS-PAGE runs as a protein of 26 kDa.

Proteolytic processing is required at both the N and C termini for activation of these cytolytic toxins (1, 3, 14). Additional processing at the C terminus results in increased toxin activity (1). The cytolytic toxin from *B. thuringiensis* subsp. *jegathesan* also requires proteolytic activation for increased biological activity. Tryptic digestion of the Cyt2Bb protein decreased its size by about 2 kDa, and N-terminal sequencing shows that N-terminus cleavage occurs right after arginine-27 of Cyt2Bb (Fig. 1 and 2B). Further tryptic digestion of the Cyt2Bb toxin by itself is not sufficient for hemolytic activity, and chymotrypsin digestion is required for hemolytic activity (18). This chymotryptic cleavage of the Cyt2Bb toxin resulted in a further 1-kDa reduction in size (data not shown). Although we do not know whether this additional processing occurs at the N terminus, it is likely to involve some C-terminus cleavage as observed with the Cyt1Aa and Cyt2Aa toxins (1, 14, 24). Nevertheless, for full activation of the Cyt2Bb toxin, both trypsin and chymotrypsin activation were required. Under these conditions, the activated Cyt2Bb toxin showed activity comparable to that of the Cyt1Aa toxin (Fig. 5).

N-terminus tryptic cleavage at an arginine residue, arginine-25, is also observed in Cyt1Aa (3, 14). The tryptic site for the Cyt2Aa toxin, which is related to the Cyt2Bb toxin, is not known but probably occurs at lysine-29. As observed with the Cyt2Bb toxin, tryptic processing of Cyt2Aa does not fully activate this toxin and additional processing with enzymes like proteinase K is required (24).

The intact toxin crystal purified from *B. thuringiensis* subsp. *jegathesan* contains a small amount of protein of 26 kDa, with most of the cytolytic toxin probably occurring as a 24-kDa proteolytically cleaved protein (Fig. 3A, lane 2). When the cloned Cyt2Bb toxin is expressed in the acrySTALLIFEROUS strain of *B. thuringiensis* subsp. *kurstaki*, most of the expressed protein migrates as a 26-kDa protein (Fig. 3A, lane 1). Hence, there is significantly less proteolytic processing of this protein in the *cryB* mutant strain of *B. thuringiensis* subsp. *kurstaki* than there is in *B. thuringiensis* subsp. *jegathesan*.

No visible crystals were observed in the *B. thuringiensis* subsp. *kurstaki* *cry* mutant cells transformed with the cloned *cyt2Bb* gene, even though the toxin is present in the spore-crystal mixture, as indicated by SDS-PAGE (Fig. 3A, lane 1). Additional attempts are now underway to increase the expression of the Cyt2Bb toxin by using the chaperonin-like 20-kDa protein of *B. thuringiensis*, which results in increased stability of the Cyt1Aa cytolytic toxin (5, 38).

In the wild-type strain *B. thuringiensis* subsp. *jegathesan*, the 26-kDa cytolytic protein is present in very small amounts (Fig. 3A, lane 2). In contrast the Cyt1Aa toxin is the most abundant protein observed in crystal preparations of *B. thuringiensis*

subsp. *israelensis* (3); and Cyt2Aa is a major protein observed in crystal preparations from *B. thuringiensis* subsp. *kyushuensis* (15, 17, 22). It is not known whether the genetic elements responsible for the expression, processing, and crystal formation of the Cyt2Bb toxin are impaired or lacking in *B. thuringiensis* subsp. *jegathesan*. Therefore, it is possible that increasing Cyt2Bb toxin expression in this strain will result in enhancement of its mosquitocidal activity due to the synergistic effect of cytolytic toxins (5, 7, 39).

The cytolytic toxins are by themselves not highly mosquitocidal (1, 5, 7, 23), and deletion of the Cyt1Aa toxin from *B. thuringiensis* subsp. *israelensis* does not decrease mosquitocidal activity (8). However, to date all mosquitocidal strains of *B. thuringiensis* reported contain a cytolytic toxin (9, 18, 22, 27, 32, 40). The biological and evolutionary significance of their presence in these mosquitocidal strains is not known. Nevertheless, the cloned cytolytic toxins act synergistically with other mosquitocidal toxins (5, 7, 39) and can also overcome mosquito resistance to the Cry1Aa toxin (10a) and also delay mosquito resistance development to *B. thuringiensis* subsp. *israelensis* (10). Consequently, the presence of a cytolytic toxin in *B. thuringiensis* formulations used for mosquito control is crucial to delay or even prevent resistance development in mosquitoes.

Five additional complete sequences of cytolytic toxins are available in the GenBank database, version 99.0, and these together with the Cyt2Bb sequence were aligned with the MegAlign computer program. Short stretches of amino acids that are highly conserved were observed. Comparison of these sequences with the three-dimensional structure of the Cyt2Aa toxin (25) showed that conserved regions are observed both in the loop regions between the α -helices and β -sheets and in the α -helix and β -sheet structures themselves. One conserved region containing the amino acids TFTNL is observed in the loop region between α -helix and β -sheet 4. It has been reported that mutations of charged residues around this region affect cytolytic activity without significant interruption of crystal formation (37), and this extended loop region may play a crucial role in various processes that lead to cell lysis. Another conserved region, QN(AGE)(QTD)TG, is observed in the loop between β -sheets 5 and 6. Based on the three-dimensional structure of the Cyt2Aa toxin (25), this region is rich in weakly charged residues and is presumed to be embedded in the membrane, possibly interacting with phospholipid-charged groups. The loop between α -helix E and β -sheet 7, with hydrophobic stretches of amino acids [LF(ILF)T(IT)], is also relatively conserved.

Additional structural conservation is observed in β -sheets 5 to 7, which can putatively form β -barrel transmembrane regions (25). The putative hydrophobic faces of these β -sheets are highly conserved, suggesting that these β -sheets probably have a functional role in pore formation and hence are conserved in all five cytolytic toxins. The minor differences in amino acid residues observed may affect the relative potency of each cytolytic toxin.

The Cyt2Bb toxin is mosquitocidal to early-second-instar larvae of *A. aegypti*, but its mosquitocidal activity is substantially lower than that of the Cyt1Aa toxin. Since we used a crystal-spore mixture for the bioassay, the exact amount of Cyt2Bb protein used cannot be determined precisely. Nevertheless, the total protein concentration and the SDS-polyacrylamide gel analysis indicate that there was more Cyt2Bb protein than Cyt1Aa protein in the same amount of the total protein used in the bioassays (Fig. 3A, lanes 1 and 4). Hence, additional investigations are needed to determine the mosquitocidal activity of purified Cyt2Bb inclusions. Though Cyt1Aa

and Cyt2Bb differ in mosquitocidal activity, they are nearly equally hemolytic.

A computer analysis of the 3' sequence of the *cyt2Bb* gene revealed two putative stem-and-loop structures with low ΔG values (-2.40 and -3.60 kcal/mol): a 9-base stem structure starting at nucleotide 1203 and a 10-base stem structure starting at nucleotide 1338, respectively. These low ΔG values suggest that additional downstream genes may be present in the same transcript. Investigations are underway to determine whether the *cyt2Bb* gene is part of a polycistronic transcript.

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