Molecular Cloning and Characterization of a Novel Mosquitocidal Protein Gene from *Bacillus thuringiensis* subsp. *fukuokaensis*

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A novel mosquitocidal protein gene, *cry20Aa*, was cloned from *Bacillus thuringiensis* subsp. *fukuokaensis* (H-3a: 3d: 3e). The gene product, Cry20Aa, was naturally truncated and had a molecular mass of 86,138 Da. The Cry20Aa protein possessed five conserved sequence blocks, as do most other insecticidal Cry toxins. However, an amino acid comparison of Cry20Aa with other mosquitocidal toxins, including Cry4A, Cry4B, Cry10A, Cry11A, and Cry11B, demonstrated that Cry20Aa was quite different from other toxins except for the conserved blocks. The N terminus of Cry20Aa was, however, homologous to the N termini of Cry4A and Cry10A. Interestingly, an inverted repeat (IR1) sequence in the open reading frame of the *cry20Aa* gene caused incomplete expression of Cry20Aa. When this internal IR1 sequence was altered with no change of amino acid sequence, acrySTALLIFEROUS *B. thuringiensis* cells transformed with *cry20Aa* gene dramatically produced crystal inclusions. However, the intact 86-kDa Cry20Aa protein is highly labile, and it is rapidly degraded to polypeptides of 56 and 43 kDa. To increase expression of the *cry20Aa* gene, the p20 chaperonelike protein and the *cyt1Aa* promoter were utilized. While p20 did not increase Cry20Aa expression or stability, chimeric constructs in which the *cry20Aa* gene was under control of the *cyt1Aa* promoter overexpressed the Cry20Aa protein in acrySTALLIFEROUS *B. thuringiensis*. The expressed Cry20Aa protein showed larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus*. However, the mosquitocidal activity was low, probably due to rapid proteolysis to inactive 56- and 43-kDa proteins.

Bacillus thuringiensis is an endospore-forming, gram-positive, soil bacterium that upon sporulation produces crystalline, proteinaceous inclusions that are toxic mainly to lepidopteran, coleopteran, and dipteran insects (4, 19). Since *B. thuringiensis* is insecticidal, there has been great interest in its use for the control of numerous agricultural pests and human disease vectors (15, 18, 21). The crystalline toxic proteins, δ -endotoxins, are predominantly synthesized as large, inactive protoxins that are activated by proteolysis in the insect gut (17). For example, Cry1, Cry4A, and Cry4B have molecular weights of 130,000 to 140,000 and are processed to active 65- to 70-kDa toxins (17, 19). While Cry2A, Cry3A, Cry10A, and Cry11A are naturally truncated toxins and have molecular weights that range from 65,000 to 80,000 (19). Proteolytic cleavage at the N and C termini can also process these naturally truncated toxins to active 60- to 65-kDa toxins, as observed in Cry2A and Cry3A (3).

Since the first cloning of the *cry1Aa* gene from *B. thuringiensis* subsp. *kurstaki* HD-1 (23), more than 100 *B. thuringiensis* toxin genes have been cloned (9). The vast majority of the toxins cloned are active against lepidopteran and coleopteran pests in agriculture. In contrast, the numbers of toxins that are active against pests of importance to human or animal health, as well as major dipteran pests of agriculture, are limited. Furthermore, only *B. thuringiensis* subsp. *israelensis*, which produces Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa toxins, is presently used for the control of mosquitoes and black flies. However, laboratory selection of *Culex quinquefasciatus* with toxins from *B. thuringiensis* subsp. *israelensis* suggests that development of mosquito resistance to individual toxins can occur (10, 16), as previously observed in lepidopterans (24). Consequently, if single toxin genes or a small subset of *B. thuringien-*

sis subsp. *israelensis* toxin genes are cloned into other organisms and used intensively for mosquito control, mosquitoes may become resistant in the field. Therefore, the isolation and characterization of novel mosquitocidal toxins are critical both for increasing the diversity of dipteran toxins and for overcoming potential problems associated with resistance.

In our continuing search for novel toxins against dipteran pests, we cloned a novel protein gene from a mosquitocidal strain of *B. thuringiensis* subsp. *fukuokaensis*. Here we report the cloning and characterization of a gene that encodes an 86-kDa protein designated Cry20Aa. We also describe the overexpression of the *cry20Aa* gene with the *cyt1Aa* promoter in an acrySTALLIFEROUS *B. thuringiensis* strain.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. thuringiensis* subsp. *fukuokaensis* H-3a: 3d: 3e, *B. thuringiensis* subsp. *israelensis* H-14, and an acrySTALLIFEROUS mutant (YG1) of *B. thuringiensis* subsp. *israelensis* used in this study have been described previously (31). In addition, an acrySTALLIFEROUS mutant of *B. thuringiensis* subsp. *fukuokaensis* (LG101) was obtained by growing the wild type in nutrient broth in the presence of 1 μ g of ethidium bromide per ml at 42°C for 24 h. Strain DH10B (Gibco-BRL) was used for cloning in *Escherichia coli*. Shuttle vector pHT304 (2) that contains both *B. thuringiensis* and *E. coli* replication origins was used for expression in *B. thuringiensis*.

N-terminal sequencing. The N-terminal sequence of an 86-kDa protein from *B. thuringiensis* subsp. *fukuokaensis* was determined as follows. Briefly, *B. thuringiensis* subsp. *fukuokaensis* inclusions were solubilized in 50 mM NaCO₃ (pH 10.0)–10 mM dithiothreitol at 37°C for 30 min and separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) gel. The gel was then transferred to a polyvinylidene fluoride membrane (Millipore). The 86-kDa protein band stained with Coomassie brilliant blue G250 was excised and subjected to N-terminal amino acid gas phase sequencing at the Biotechnology Instrumentation Facility at the University of California, Riverside. The N-terminal sequences of the proteolytically processed 56- and 43-kDa proteins were similarly determined.

PCR identification and isolation of the 86-kDa protein gene. A degenerate 32-mer oligonucleotide, BTFN32, used for the forward PCR primer was designed based on the N-terminal sequence of the 86-kDa protein (Table 1). A degenerate 27-mer oligonucleotide, BTCB3, was constructed from conserved sequence block 3 in Cry4A, Cry4B, and Cry10A toxins and was used as the reverse PCR primer. For PCR, 0.1 μ g of total DNA from *B. thuringiensis* subsp.

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence ^a
BTFN32 ^b	ATGAAC(T)CCITA(C/T)CAA(G)AAC(T)AAIAAC(T) GAAATTGT
BTCB3 ^c	IAA(G)A(G)TCICCCIGTA(G)TGNCNGGNCC
BTFN27S	AATACGAATTATAAAGATTGGATGAAT
BTFN27A	TGCATAGTTTGGTAATAAAAGAGTTTC
BTF2655S	TCAATCAATTCTGGGAATTTTAAAGAGATAC
BTF2655A	TTCCCAGAATTGATTGAGAACCCATCTTGAAA
BTF3640NHE	CTGTGCTAGCAGTATCATTAGATTTACA ^d
BTF1856STU	TTTCAGGCCTCATAATATACCAAGGTAT ^e
HINCYTA-1S	TTACAAGCTTCCCAATACCAATTCTAA ^f
CYT86-2S	AAGGAGTTGTTTATTTATGAATCCGTATCAAAAT
CYT86-2A	TATTTTGATACGGATTTCATAAATAACAACCC
BSR86-1A	AGCGTGATACAATGAGTGATATATTCG ^g
SALIVD-1S	ACTAGTCGACAATGATACGTTTGGATATC ^h
20IV-2S	AGGGAGAGAAGATAAATATGACAGAAAATGGA GTG
20IV-2A	CACTCCATTTTCTGTCATATTTATCTTCTCTCCCT
SAL20-1A	ACCCGTCGACTAATGTTTCCAGTGCATT ^h

^a I, inosine; N, guanine, adenine, thymine, or cytosine.

^b Degenerate primer based on the N-terminal sequence (MNPYQNNNEIV) of the 86-kDa protein.

^c Degenerate reverse primer for conserved sequence block 3 (GPGHTGGDL) of the Cry4A, Cry4B, and Cry10A toxins.

^d The *NheI* restriction enzyme site is underlined.

^e The *StuI* restriction enzyme site is underlined.

^f The *HindIII* restriction enzyme site is underlined.

^g The *BsrGI* restriction enzyme site is underlined.

^h The *SalI* restriction enzyme site is underlined.

fukuokaensis was mixed with a solution containing each primer at a concentration of 1 μ M, each deoxynucleoside triphosphate at a concentration of 200 μ M, and 2.5 U of *Taq* polymerase in PCR buffer (Gibco-BRL). PCR amplification was accomplished with a DNA thermal cycler (Perkin-Elmer Cetus) by using the following program: 94°C for 1 min, 47°C for 2 min, and 72°C for 3 min for 30 cycles.

Isolation of the wild-type *cry20Aa* gene. Total DNA of *B. thuringiensis* subsp. *fukuokaensis* was digested with different restriction enzymes, separated in a 0.6% agarose gel, and then transferred to a nylon transfer membrane (Micron Separations Inc.). The membrane was then probed with a ³²P-labeled probe made with random hexamers by using the 2.7-kb PCR fragment that encodes full-length 86-kDa protein as the template and amplified as described above. Hybridization was performed at 42°C for 16 h by using 10⁷ cpm/ml. The membrane was washed at 65°C in solutions containing 2 $\times$ to 0.2 $\times$ SSC and 0.2% SDS (1 $\times$ SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.4).

Total DNA from *B. thuringiensis* subsp. *fukuokaensis* was digested with *HindIII* and *PstI*. Fragments that hybridized to the 2.7-kb PCR fragment were eluted from the agarose gel by using a Sephaglas kit (Pharmacia) and were ligated into the *HindIII*-*PstI*-digested pHT304 vector (2) to make a plasmid library. The library was then screened by limited growth PCR (22) by using a set of nested primers, BTFN27S and BTFN27A, which were designed by using the 2.7-kb PCR fragment (Table 1).

The 6.5-kb *HindIII*-*PstI* insert in pBTF86HP (Fig. 1) was further digested with

ScaI, and the resulting 3,586-bp *HindIII*-*ScaI* fragment was then subcloned into the pHT304 vector restricted with *HindIII*-*SmaI*. Both strands of the clone were sequenced by using Sequenase 2.0 as described by the manufacturer (Amersham) and primer walking.

Protein expression in *B. thuringiensis*. For gene expression, acrylamidiferous mutant cells of *B. thuringiensis* subsp. *israelensis* (YG1) or *B. thuringiensis* subsp. *fukuokaensis* (LG101) were utilized. Briefly, bacterial cells cultured in 500 ml of brain heart infusion broth to an optical density at 600 nm of 0.4 were centrifuged, washed three times with 10% glycerol, and then resuspended in 0.8 ml of 40% polyethylene glycol 8000. The cells suspended in polyethylene glycol 8000 (0.2 ml) were mixed with 0.5 μ g of plasmid DNA, and electroporation was performed in a 0.2-cm cuvette by using a Bio-Rad Gene Pulser apparatus set at 25 μ F, 2.5 kV, and 400 Ω . The transformants were selected on nutrient agar supplemented with 50 μ g of erythromycin per ml at 30°C, and the formation of parasporal inclusions was observed by phase-contrast microscopy.

B. thuringiensis transformants were grown on nutrient agar plates supplemented with 50 μ g of erythromycin per ml until cell autolysis was observed. The lysates were then harvested and washed with 10 mM EDTA–1 M NaCl–0.1 mM phenylmethylsulfonyl fluoride to partially remove the spores. The parasporal inclusions of *B. thuringiensis* subsp. *fukuokaensis* and *B. thuringiensis israelensis* were purified on discontinuous NaBr gradients as described previously (5). Protein concentrations were determined with a detergent-compatible protein assay kit (Bio-Rad) by using bovine serum albumin as the standard.

Immunoblot analysis. Proteins were separated on a 10% SDS–PAGE gel and then transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 2 h in phosphate-buffered saline (PBS) containing 5% bovine serum albumin and then probed for 1 h with rabbit antibody developed against whole inclusion proteins of *B. thuringiensis* subsp. *fukuokaensis* (31) in PBS–0.05% Tween 20. The membrane was washed three times with PBS–0.05% Tween 20 and then incubated with goat anti-rabbit immunoglobulin G–alkaline phosphatase. The membrane was then incubated with the substrate, a solution containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Internal stem-loop modification. An internal stem-loop that consists of a 16-base stem and a 10-base loop between bp 2656 and 2697 of the *cry20Aa* gene was modified (Fig. 2). Two products were amplified by Expand PCR (Boehringer Mannheim); these products were a 799-bp fragment between the *StuI* site and the inverted repeat 1 (IR1) site (amplified by using the BTF1856STU and BTF2655A primers [Table 1]) and a 985-bp fragment between the IR1 site and the *NheI* site (amplified by using the BTF2655S and BTF3640NHE primers [Table 1]). The two products were then fused and further amplified by using the BTF1856STU and BTF3640NHE primers. The amplified fusion product (1,784 bp) was digested with *StuI*-*NheI* and then used to substitute for the *StuI*-*NheI* fragment flanking the internal IR1 site of the *cry20Aa* gene. The modified *cry20Aa* gene in plasmid pBTF86HS was then sequenced to confirm that no mutations were introduced.

Chimeric constructs. The *cyt1Aa* gene promoter (27, 28) was amplified by using Expand PCR and two primers (Table 1), a forward primer (HINCYTA-1S) containing a *HindIII* site and a reverse primer (CYT86-2A) that includes a 16-base sequence from the ATG start codon of the 86-kDa protein gene. Similarly, a 698-bp PCR fragment between the ATG start codon and the *BsrGI* site in the 86-kDa protein gene was amplified with two primers (Table 1), a forward primer (CYT86-2S) that includes a 16-base sequence of the *cyt1Aa* promoter downstream and a reverse primer (BSR86-1A) containing a *BsrGI* site. Both PCR products were first denatured and annealed and then further amplified by using both HINCYTA-1S and BSR86-1A. The amplified 1,086-bp fusion product was digested with *HindIII*-*BsrGI* and used to replace the *HindIII*-*BsrGI* fragment of the wild-type 86-kDa protein gene (Fig. 1).

In the same way, the full-length copy of the 20-kDa chaperonelike protein (p20) gene from *B. thuringiensis* subsp. *israelensis* (1) was amplified by using Expand PCR and fused with the *cry11A* (*cryIVD*) gene promoter (13). Briefly, the

FIG. 1. Restriction map of recombinant plasmid pBTF86HP, which contains the *cry20Aa* gene. The thin bars represent noncoding regions of the 6.5-kb insert. The open boxes represent shuttle vector pHT304. The arrow indicates the orientation of the *Cry20Aa* gene. IR1 and IR2 indicate the positions of inverted repeats 1 and 2, respectively.

1 AG CTT GTC TCA CTT GAA ATA AGC TCA CCA TTA CGT TTT TGT TTA CAA GGT AAC GGT ACA GTT TTC GAT AGC CCA TTC TAC CTT
84 TTA AAC GTT TAA ATT GCA TAA CAA TAT AAG CCG CTT TCT TTT TAC GTT TTT TCT GAT TTT CAG TGG GCT TAC GAT GAA TAT AAT
168 CGT AAA AAC CAC TAA CAG AAA CTT GTA ATC GAT GGC AAA GCA GGC CAC TCC AAA TAT GTG ATG ATA TTG CCT GAT AAA TTG AAA
252 GCG TGT TAG TTT TGG TTG CTG GCA AAG AAG GTT GCC GCC TTT TTT AGA AAT TCA TTT TCC TTT TTT AAA GCC TGG ATT TCT CTC
336 CGC AAA GAA CGT AGT TCT TCG ATA TCA GAT TTC TCA TTA TCC AAA AAT GGG GAA GAA TAT TTC TTT ATC CAT GCA TTT AGG GTC
420 CGT TCC CTA GGT CAA ATT CTT TTT GAA TAC TGG AAA TTG TAC GCC CTT CTT TGA CCA CTA ATT CTA CAC ATT GTT TTT TAA ACA
504 TTT CAT CAT ACT GTT TTT TCA TCT GAA ACA CCT CTT ATC TAG AAT AAT TTA TCT CTA TAC TGT GTT CCA GTT TTA CTG TAC CAC
588 CTC ACA ATA TAA ACG AAA AGG AAG TTT GCA ACA ACC AAA CTT CGT TTT TTC CAC ATT CAA TGA ACT ACA ACA ATT ACT AGC CAC
672 TGC TTA ACT ATT TCT CTT CAT TTG CCA ACT TGT TTA TCC TTT TTT CAA ACT TTG CAA AAG AAC CCA GGA GTA CAT AGG GGC ATT
756 ACG AAT ATT TAA AAA GAC ACA GGC ATA TAT TTG TAT GGT GGG ATG AAG GAT AAG AAA TGA GAA GAA TAC GAG GGA GGA ACA ATA
SD
840 ATG AAT CCG TAT CAA AAT AAC GAT GAA ATA GTA GAT GTT CCG GAA AAT TAT GAC AAT AAC TTA AAT AGG TAT CCT TAC GCA AAT
M N P Y Q N N D E I V D V P E N Y D N N L N R Y P Y A N 28
924 GAT CCA AAT GTC GCA AAT ACG AAT TAT AAA GAT TGG ATG AAT GGG TAT GAA GAA ATT AAT CCT TCT TCA ATA ATT GCA
D P N V T N Y K D M N G Y E E I N P C S T S A I T A 56
1008 ATT TTA GCT TCA ATA GGA ATT CTT AAT AGG GTA ATT GCT TTA ACT GGA GTA TTA GGT AAT ACA CAA GAA GTT ATT AGT ATA ATC
I L A S I G I L N R V I A L T G V L G N T Q E V I S I I 84
1092 CAA GAT GCA TTG GGA TTT ATT CGA AAT GGA ACA GGG AAT GAA TTA TTA ATT CAT GTA GAA CAA CTT ATT CAG CAA ACA TTA GCA
Q D A L G A M G T F I R N G T A G G G A A T L L L I H V E Q L I Q I T G L A 112
1176 ACA CAG TAT AGG AGT GCT GCA ACT GGA GCC ATA TAT GGC ATA TCT AGA TCA TAC GAT AAT TAT TTG ATG TTT TTT CAG CAA TGG
T Q Y R S A A T G A I Y G I S R S Y D N Y L M F F R Q W 140
1260 GAA CGT AAT AGA ACT CGT GAA AAC GGA CAA CAA GTA GAG AGT GCT TTT ACT ACT ATT AAT ACT TTA TGT ATT AAT GCT TTA GCT
E R N R T R E N G Q Q V E S A F T T I N T L C I N A L A 168
1344 CCT CAG GCA TCA CTT TCT CGC AGA GGA TTC GAA ACT CTT TTA TTA CCA AAC TAT GCA ATG GCG GCA AAT TTC CAT TTG TTA TTA
P Q R A S L S R A F E T L L L P N Y A M A A N F H L L L 196
1428 TTA AGA GAT GCT GTT CTT TAT AGA AAT CAA TGG TTA TCT AAT TCT ATT TCA ACA GCA AAT GTG AAT CTT AAT ATA TTG AGA GCT
L R D A V L Y R N Q W L S N S I S T A N V N L N I L R A 224
1512 GCC ATA AAC GAA TAT ATC ACT CAT TGT ACA CGC TGG TAT CAA GAT GGT CTG AAC AGA TTT GAT CGT TCA AGT CGT GCA AAT ATG
A I N E Y I T H C T R W Y D G L N R F D R S S R A N M 252
1596 AAT GAG TGG AGA AGG TTT AAT GCG TAT CGT AGG GAT ATG ACG TTG TCC GTA TTA GAC TTC GCT ACA GTA TTT CCA ACT TAT GAT
N E W R R F N A Y R R D M T L S V L D F A T V F P T Y D 280
1680 CCT GTT CTA TTT CCA GCA GCA ACA AAT GTT GAA TTG ACT AGA GTC GTT TAT ACA GAT CCA ATA GTG ATG GCA GGG GGA AGG ACA
P V L F P A A T N V E L T R V V Y T D P I V M A G G R T 308
1764 GCA ATC CCT GGC TTC ACT CGT ATG GAA AAT CTA GTT AAT TCT GCT AGT AGA GTT TCT TTT TTA AAT CAG ATG AAT ATA TAT ACA
A I P N S E L V N A S A S R V S F L N Q M N I Y T A 336
1848 AGT TTT TAT TTC AGG CCT CAT AAT ATA CCA AGG TAT TAT TGG TCT GGT AAT CAG AAT TTT TTA AGT AAC GGA ACT TCG AAT TTA
S F Y F R P H N I P R Y Y W S G N Q N F L S N G T S N L 364
1932 TAT GGA TAC AGA TCG GAT GGA CGG ACT ACG TTT AAT GTG AGC AAT ATT GAT ATT TTC AGA GTA AAT ATG ACA ACT CAT ATA GGT
Y G Y R S T F N V S N I D I F R V N M T H I A G 392
2016 GGT GCT TTT ACA GAT GAC TAT CGT GGA CTT CAT AGG GCT GAG TTT ATT GGT GCA AAT ACA CAA AAT AAT CAA AGA ACA TCA CTA
G A F T D D Y R G L H R A E F I G A N T Q N N Q R T S L 420
2100 TTA TAT AGC GTA GAG ATT CCT TCT TCT CAC TTT AGA TTT GAG AAT CAT ACG GTA TTT TTA CCA GGC GAG TCA GGA TTA GAA CCA
L Y S V V E I T S H F R F N H T V F L P G E S G L E A P 448
2184 AAT GAA CGT AAT TAT ACT CAT AGA TTA TTT CAA ATG ATG AAT GAG GTT TCT GTT AAT CCT AAT GCC GCG GGA CGA GTT TTT TTA
N E R N Y T H R L F Q M M N E V S V N P N A R G R V F L 476
2268 CAT GCA TGG ACG CAT AGA AGT TTA AGA CGT ACA AAT GGA TTG AGG TCG GAT CAG ATT CTG CAA ATA CCA GCT GTA AAA ACC ATA
H A W T H R S L R R T N G L R S D Q I L Q I P A V K T I 504
2352 AGT AAT GGT GGT GAT CGT GCT GTA GTG TTA AAT TAC GGA GAA AAC ATT ATG AAA TTA GAT AAT TTA ACT ACA GGT TTA TCC TAT
S N G R A V V L N Y G N I M K L D N L T T G L S Y 532
2436 AAA TTA ACA GCC GTG GAT TCA GAA GCG TCT AAT ACA CGT TTT ATT GTG CGT GTT CGT TAT GCT AGT ATG AAC AAT AAT AAA TTG
K L T A V D S E A S N T R F I V R V R Y A S M N N N K L 560
2520 AAT CTT GTT TTA AAT GGT GCT CAG ATA GCA TCA CTG AAT GTG GAA CAT ACA GTA CAA CGT GGA GGA TCA TTA ACG GAT CTT CAA
N L V L N G A Q I A S L N V E H T V Q R G G S L T D L Q 588
IR1
2604 TAT GGA AAT TTT AAA TAT GCT ACA TTT GCA GGT AAT TTC AAG ATG GGT TCT CAG TCT ATA TTA GGT ATT TTT AAA GAG ATA CCT
Y G N P K Y A T F A G N F K M G C Q S I L G I F K E I P 616
2688 AAT ATA GAC TTT GTC TTA GAT AAA ATT GAA TTG ATT CCA TCG AAT TTC ATG TCA TTA GAG CAA ACA AAT TAT EAT AAC ACC
N I D F V L D K I E L I P S N F M S S L E Q T Q N Y N T 644
2772 TAT AAT CAA GAT ACT ATT TAT ACA CAT AAC CAA GGA TAT GAT ACT TAC GAT CAG AAT TCT AGT GGT ATG TAT CAT CAA AGC TAC
Y N Q D T I Y T H N Q G Y D T Y D Q N S S G M Y H Q S Y 672
2856 AAC AAT TAC GAT CAA AAC ATG GAT ACT ACG TAT CAA CCA AGC TAC GAT AAT TAT AAT CAG AAT GCC AGT GGT ACG TAT GAT GAT
N N Y D T Q N M D T T Y Q P S Y D N Y N A S G T Y D D 700
2940 GGG TAT AAC CCA AAT GCC AGT GAT AGT TAT GAT CAA AGT TAT ACC AAC AAT TAT AGT CAG AAT ACT AAC AGT ATG TAT GAT CAA
G Y N P N A S D S Y D Q S Y T N N Y S Q N T N S M Y D Q 728
3024 GGT TAT TAT AAT AAC AAT TAT GAC CAG CAT TCC GGT TGT ACG TGT AAC CAA GGG TAT GAC AAT AAC TAT CTA AAA TAA GAA CAA
G Y Y N N Y D Q H S G C T C N Q G Y D N N Y L K * 753
3108 GAA AGA GTA TCC TCA CTC ACC AGG GGA ATG CTC TTT TGT TGT TTA TTT CAC ACT ATC CCT CAA ACC TTA CAT ACT CCC CTG ATA
3192 CCC ATT GAT CTC CAC TAA CGT TAT ACC ATC TTT TTA TGC CCT ACG ATT GAT ATC GTA CCT TTT TTA CAA TTT CGT AAC TTG
3276 TTC CCG GCC CTG TAC GAA TGC GTA CTA CAT GGT TCT GGT GCA AAA GTG TAA AAA GAC ATG AAA GTA GGA ACT AAT TTT ATA GTG
IR2
3360 TAA AAT GCT GAA TTA TTG ACG GAT ACA GTT CAT TTA GTA CCC ATT TTC TTA TCC ACA TAT CAA GAC AAG AAA ATG GGT ACT TTT
3444 TTA AAC TAA GAC AAC AAA GTG GAT ATT TTT TAA AAA TGG TAA AAC AGG CAA CTA AAT TTG TTA TAA TAA ATA AAT ATT AAA TTT

FIG. 2. Nucleotide sequence of the *cry20Aa* gene and deduced amino acid sequence of the protein. The heavy lines 5' to the ATG codon indicate putative promoter sites. A putative ribosome binding Shine-Dalgarno (SD) sequence is enclosed in a box. The arrows indicate inverted repeat (IR1 and IR2) sequences. The nucleotide substitutions for IR1 removal are indicated below the IR1 arrow. Five conserved sequence blocks (blocks 1 to 5) are underlined. The N termini of 56-kDa (LLIHVE) and 43-kDa (NVNLI) degradation products are double underlined.

cry11A promoter was amplified with two primers (Table 1), a forward primer (SALIVD-1S) containing a *Sal*I site and a reverse primer (20IVD-2A) that includes a 16-base sequence from the ATG start codon of the *p20* gene. A 678-bp PCR fragment that includes the ATG start codon and the inverted repeat termination of the *p20* gene was amplified with two primers (Table 1), a forward primer (20IVD-2S) that includes a 16-base sequence of the *cry11A* promoter and a reverse primer (SALIVD-1A) containing a *Sal*I site. Both PCR products were fused and further amplified by using SALIVD-1S and SALIVD-1A. The resulting 1,036-bp chimeric *p20* gene in pLG1 contains *Sal*I sites at both the 5' and 3' ends. All chimeric constructs were further sequenced to confirm that the proper constructs were obtained and that no mutations were introduced.

Insect bioassay. Mosquitocidal activity was tested in 10 ml of tap water with 10 second-instar larvae of *Aedes aegypti* and *C. quinquefasciatus*. The assays were performed in triplicate by using three doses, and mortality was scored after incubation for 24 and 48 h at 22°C. The entire bioassay was repeated once, and 50% lethal concentrations (LC₅₀s) were determined by averaging the results from these determinations.

Nucleotide sequence accession number. The nucleotide sequence accession number for *cry20Aa* is U82518.

RESULTS AND DISCUSSION

The first 11 amino acids of the 86-kDa protein from mosquitocidal *B. thuringiensis* subsp. *fukuokaensis* (31) were determined by protein sequencing to be MNPYQNNNEIV. This N-terminal sequence was highly homologous to the N terminus of the Cry4Aa and Cry10Aa toxins, MNPYQNKNEYE (25, 29). To isolate the gene that encodes this 86-kDa protein, we first attempted to isolate a fragment by PCR by using primers BTFN32 and BTCB3 (Table 1). When a PCR was performed with total DNA of *B. thuringiensis* subsp. *fukuokaensis*, no product was detected. However, a 2.7-kb PCR product was obtained when only the BTFN32 primer was used in the PCR. This 2.7-kb product was not obtained when total DNA from a plasmid-cured mutant of *B. thuringiensis* subsp. *fukuokaensis* was used for PCR. Nucleotide sequencing of this PCR product showed that it encodes the 86-kDa protein; however, Asp-8 of the N terminus was incorrectly determined to be Asn-8 during protein sequencing (Fig. 2).

Southern analysis showed that this 2.7-kb PCR product hybridized to a 5- to 7-kb band of *Hind*III-*Pst*I-restricted total DNA from *B. thuringiensis* subsp. *fukuokaensis*. This band was then used for construction of a plasmid library. Screening of this library led to the isolation of a clone that contained a 6.5-kb insert (Fig. 1). The insert in this plasmid, pBTF86HP, was subjected to restriction enzyme analysis and nucleotide sequencing.

The DNA insert contains a 2,260-bp open reading frame (ORF) that encodes a 753-amino-acid polypeptide with a molecular mass of 86,138 Da (Fig. 2). The Cry20Aa protein contains the five conserved sequence blocks that are characteristic of other toxins, such as Cry1, Cry4, and Cry10 (19), but belongs to a new class of Cry proteins, the Cry20Aa class. Not surprisingly, the nine conserved residues, GPGHTGGDL, in block 3 of Cry4A, Cry4B, and Cry10A, which were used for designing the BTCB3 primer (Table 1), were not found in Cry20Aa (Fig. 2).

This ORF is flanked by 839 bp upstream and 3,401 bp downstream (Fig. 1). A putative ribosome binding site, which corresponds to a *Bacillus* Shine-Dalgarno sequence (20), is located seven bases upstream from the start codon (Fig. 2). An inverted repeat (designated IR2) with a ΔG° of -21.7 kcal (26), which may act as a transcription terminator, is located 293 bases downstream from the first stop codon (Fig. 2). Interestingly, inverted repeat (designated IR1) with a ΔG° of -20.2 kcal is found within the ORF between conserved sequence blocks 4 and 5 (Fig. 2).

The deduced amino acid sequence of Cry20Aa was compared with the sequences of other mosquitocidal toxins, such

as Cry4Aa (29), Cry4Ba (8), Cry10Aa (25), Cry11Aa (14), and Cry11Ba (12). Cry20Aa has limited similarity to Cry4A, Cry4B, and Cry10A, mostly in the regions of the conserved sequence blocks, with overall levels of amino acid identity of 21 to 23%.

Initial attempts to express the Cry20Aa protein by using plasmid pBTF86HP in acrySTALLIFEROUS *B. thuringiensis* subsp. *israelensis* (YG1) and *B. thuringiensis* subsp. *fukuokaensis* (LG101) mutants were not successful. Very low expression levels were observed, and these levels were detectable only by immunoblotting (data not shown).

To improve protein expression, several modifications were introduced. First, the downstream DNA sequence was deleted and the internal IR1 sequence present between conserved blocks 4 and 5 was modified (Fig. 2) to remove possible incomplete transcription without changing the amino acid residues of Cry20Aa. Second, the *cyt1Aa* gene promoter and its ribosome binding site were utilized in place of the *cry20Aa* gene promoter (Fig. 3). Third, *p20*, a 20-kDa chaperonelike protein gene under control of the *cry11A* promoter, was included in the plasmid constructs upstream of the *cry20Aa* gene (Fig. 3). Furthermore, this *p20* protein gene was introduced in both orientations relative to the *cry20Aa* gene, since we have previously observed some differences in the expression of Cry proteins when the orientation is changed.

By using the wild-type inverted repeat-modified Cry20Aa, the chimeric Cry20Aa, and *p20* protein genes, a series of plasmids were constructed (see Fig. 3 legend for details). These plasmids, pBTF86HS, pLG201, pLG202, pLG203, pLG204, and pLG205, were used to determine Cry20Aa expression in YG1 cells. In all of these transformations inclusions were visible when phase-contrast microscopy was used (Fig. 4). Interestingly, inclusions were also observed when plasmid pBTF86HS, which has a modified internal inverted repeat, was used in the transformations. This data suggests that the internal inverted repeat, IR1, may affect gene transcription either by decreasing transcript stability, by decreasing transcription, or by forming incomplete transcripts that fail to produce sufficient levels of protein to form inclusions. However, detection of inclusion formation and Cry20Aa production demonstrates that the promoter for the *cry20Aa* gene is within the 838-bp upstream sequence (Fig. 2).

Large inclusions were observed in YG1 cells that were transformed with plasmid constructs pLG201, pLG204, and pLG205, in which the *cry20Aa* gene was under control of the *cyt1Aa* promoter (Fig. 4). This promoter is activated by transcription factors $E\sigma^{35}$ and $E\sigma^{28}$ through early and late sporulation in *B. thuringiensis* subsp. *israelensis* (27, 28), resulting in high Cyt1Aa protein expression, which consists of more than 50% of the total parasporal inclusion proteins in *B. thuringiensis* subsp. *israelensis* (27, 28). However, all transformants with plasmids that utilized the *cyt1Aa* promoter had difficulty sporulating, possibly because the *cyt1Aa* promoter affects other processes that are dependent on sporulation-specific sigma factors.

Expression of Cry20Aa was further analyzed by SDS-PAGE and immunoblotting. *B. thuringiensis* YG1 cells transformed with each of the plasmids described above were harvested at the late sporulation stage at ca. 48 h. The spore-inclusion mixtures were directly analyzed on a 10% polyacrylamide gel (Fig. 5). Protein expression was lower when plasmids pBTF86HS, pLG202, and pLG203, in which the *cry20Aa* gene is under control of its own promoter, were utilized (Fig. 5, lanes 2, 4, and 5, respectively). Higher protein expression was detected when plasmids pLG201, pLG204, and pLG205 were used (Fig. 5, lanes 3, 6, and 7, respectively).

FIG. 3. Subclone and chimeric constructs of the *cry20Aa* gene in shuttle vector pHT304. pBTF86HS, a subclone, contains the *Hind*III-*Sca*I fragment from the pBTF86HP clone and a modified IR1 sequence. The *cry20Aa* gene is under control of the native Bt86 promoter. In pLG201, the *cyt1Aa* promoter is fused with the *cry20Aa* gene, which also contains a modified IR1 sequence. pLG202 and pLG203 are pBTF86HS variants that have in addition, in opposite orientations, the *p20* gene fused with the Cry11A promoter. pLG204 and pLG205 are pLG201 variants that have in addition, in opposite orientations, the *p20* gene fused with the Cry11A promoter. The open boxes represent shuttle vector pHT304. The arrows indicate transcriptional orientation. prom, promoter.

However, irrespective of the expression levels, the full-length 86-kDa protein was rapidly degraded to smaller proteins of 56 and 43 kDa. More extensive degradation of the Cry20Aa protein was observed in YG1 cells transformed with pBTF86HS, pLG202, and pLG203, in which mostly the 43-kDa protein was detected (Fig. 5, lanes 2, 4, and 5, respectively). Extensive Cry20Aa degradation was also observed in YG1 cells transformed with pLG201, pLG204, and pLG205 (Fig. 5, lanes 3, 6, and 7). In these cells the 86- and 56-kDa bands were visible. These protein bands were visible because higher Cry20Aa protein levels were expressed and degradation was probably not complete. Furthermore, no difference in the extent of pattern of protein degradation was observed whether the Cry20Aa protein was expressed in *B. thuringiensis* subsp. *israelensis* YG1 or *B. thuringiensis* subsp. *fukuokaensis* LG101 cells (data not shown).

The 56- and 43-kDa proteins were confirmed to be the degraded products of the intact 86-kDa Cry20Aa protein by immunoblot analysis (Fig. 6, lanes 1 and 2) and also by N-terminal sequencing. The N-terminal sequences of the 56- and 43-kDa proteins were LLIVHE and NVNLNI, respectively,

showing that cleavage occurs between Glu-98 and Leu-99 and between Ala-215 and Asn-216 of the intact 86-kDa Cry20Aa protein. This cleavage pattern indicates that both the 56- and 43-kDa proteins have truncated domains I and III that are required for insecticidal activity (6, 7).

To decrease this protein degradation, the chaperonelike p20 protein under control of the *cry11A* promoter was used in combination with the *cry20Aa* gene. The p20 protein from *B. thuringiensis* subsp. *israelensis* is known to enhance protein production and help stabilize other *B. thuringiensis* proteins, such as the Cyt1Aa protein, whose expression in *E. coli* requires the p20 protein (1, 30). Although the p20 protein increases *cyt1Aa* expression in *B. thuringiensis*, it did not enhance Cry20Aa expression or its stabilization. Constructs that contained the *cry20Aa* gene exhibited similar degradation patterns regardless of p20 coexpression (Fig. 5, compare lane 2 with lanes 4 and 5 and lane 3 with lanes 6 and 7). In contrast, in *B. thuringiensis* subsp. *fukuokaensis* the Cry20Aa protein exists as an 86-kDa protein, and no 56- and 43-kDa proteins were detected (Fig. 5, lane 8). Since wild-type *B. thuringiensis* subsp. *fukuokaensis* contains a number of proteins, each protein not

FIG. 4. Phase-contrast micrographs of *B. thuringiensis* transformants that contain different plasmids. pHT304, *B. thuringiensis* cells that were transformed with only the plasmid vector, pHT304; pBTF86HS, *B. thuringiensis* cells that were transformed with plasmid pBTF86HS, whose map is shown in Fig. 3; pLG204, *B. thuringiensis* cells that were transformed with pLG204, whose map is shown in Fig. 3. Bars = 1 μ m. S, spore; C, crystal inclusion protein.

FIG. 5. SDS-PAGE analysis of *cry20Aa* gene expression in acrystalliferous *B. thuringiensis* subsp. *israelensis* YG1. Lane 1, pHT304 vector; lane 2, pBTF86HS; lane 3, pLG201; lane 4, pLG202; lane 5, pLG203; lane 6, pLG204; lane 7, pLG205; lane 8, *B. thuringiensis* subsp. *fukuokaensis* inclusion protein; lane S, standard proteins, including myosin (molecular weight, 205,000) β -galactosidase (116,000), phosphorylase *b* (97,000), fructose-6-phosphate kinase (84,000), bovine serum albumin (66,000), glutamic dehydrogenase (55,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), and trypsinogen (24,000). Lanes 1 to 7 each contained 20 μ g of cell lysate; lane 8 contained 10 μ g of purified inclusion protein.

only is a component of the inclusion but may also function to stabilize other proteins. This in part may explain the Cry20Aa stability in the wild-type strain but not in plasmid-cured strain LG101.

To evaluate the mosquitocidal activity of the Cry20Aa protein, attempts were made to obtain purified parasporal inclusions. However, all attempts were unsuccessful since Cry20Aa rapidly degraded into tiny inclusions upon cell autolysis. These tiny inclusions contain mostly the smaller 56- and 43-kDa pro-

TABLE 2. Mosquitocidal activity of *B. thuringiensis* transformants

<i>B. thuringiensis</i> cells ^a	LC ₅₀ (μ g/ml) ^b	
	<i>A. aegypti</i>	<i>C. quinquefasciatus</i>
pLG204 (Cry20Aa)	648 $\pm$ 52	700 $\pm$ 50
pHT304 (vector)	>5,000	>5,000
YG1 ^c	ND ^d	ND

^a Spore-inclusion mixtures were harvested from autolyzed cells.

^b Second-instar larvae were tested, and LC₅₀s were determined at 48 h.

^c Acrystalliferous *B. thuringiensis* subsp. *israelensis* strain.

^d ND, not determined.

teins, as determined by SDS-PAGE. Cry20Aa was degraded even when protease inhibitors were used during the isolation and purification procedures. Hence, the mosquitocidal activity was determined by using crude spore-inclusion mixtures.

The evaluation of the insecticidal activity of the Cry20Aa protein against *A. aegypti* and *C. quinquefasciatus* second-instar larvae showed that the Cry20Aa protein has low mosquitocidal activity (LC₅₀, 600 to 700 μ g/ml) when a mixture of spores and crystals is used (Table 2). This low mosquitocidal activity is not surprising since very low levels of the intact 86-kDa Cry20Aa protein were present in the preparations used for the insect bioassays. The 56- and 43-kDa proteins that were present at much higher levels were not expected to be insecticidal since both of these proteins have truncated domains I and III. Domain I forms pores in the insect midgut cell epithelium and is essential for insecticidal activity (6). Similarly, domain III is increasingly thought to play an essential role in insecticidal activity (7). Interestingly, the 72-kDa Cry11Aa protein from *B. thuringiensis* subsp. *israelensis* is degraded to N- and C-terminal fragments of 40 and 32.5 kDa, respectively, by bacterial and mosquito larval midgut proteases. The individual N- or C-terminal moieties do not have mosquitocidal activity when applied individually, but mosquitocidal activity is restored when the two proteins are packaged together (11). Consequently, to obtain higher mosquitocidal activity, it is essential that the degradation of the Cry20Aa be decreased, and attempts are underway to increase the stability of this protein.

FIG. 6. SDS-PAGE and immunoblot analysis of purified Cry20Aa inclusions from pBTF86HS- and pLG204-transformed YG1 cells. The immunoblot was probed with antibody raised against solubilized crystal proteins of *B. thuringiensis* subsp. *fukuokaensis*. Lane 1, pLG204; lane 2, pBTF86HS; lane 3, *B. thuringiensis* subsp. *fukuokaensis*; lane 4, *B. thuringiensis* subsp. *israelensis*; lane 5, pHT304 vector; lane S, standard proteins as described in the legend to Fig. 5. Lanes 1 to 4 each contained 10 μ g of purified inclusion proteins; lane 5 contained 20 μ g of cell lysate.

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