Cloning and Expression of Two Crystal Protein Genes, *cry30Ba1* and *cry44Aa1*, Obtained from a Highly Mosquitocidal Strain, *Bacillus thuringiensis* subsp. *entomocidus* INA288

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Two novel crystal protein genes, *cry30Ba* and *cry44Aa*, were cloned from *Bacillus thuringiensis* subsp. *entomocidus* INA288 and expressed in an acrySTALLIFEROUS strain. *Cry44Aa* crystals were highly toxic to second-instar *Culex pipiens pallens* (50% mortality concentration [LC₅₀] = 6 ng/ml) and *Aedes aegypti* (LC₅₀ = 12 ng/ml); however, *Cry30Ba* crystals were not toxic.

Bacillus thuringiensis subsp. *israelensis* produces crystal proteins that have been successfully used for controlling the mosquito population (2). Although no resistance to *B. thuringiensis* subsp. *israelensis* toxins in the field has been reported, weak resistance has been observed in the laboratory in populations that are under artificial selection pressure due to high concentrations of *B. thuringiensis* subsp. *israelensis* toxins (3). Therefore, it is possible that field populations of insects resistant to *B. thuringiensis* subsp. *israelensis* toxins may emerge if large concentrations of *B. thuringiensis* subsp. *israelensis* toxins are used for an extended period. Hence, several screening programs have been set up that aim to isolate new strains producing novel mosquitocidal crystal proteins that could replace or be used in combination with *B. thuringiensis* subsp. *israelensis*. *Bacillus thuringiensis* subsp. *entomocidus* INA288 has been isolated from Indonesian soil, and it produces large cuboidal crystals (5). Although known mosquitocidal *cry* genes, such as *cry2A*, *cry4A*, *cry4B*, and *cry11A*, were not detected by PCR, this strain showed a toxicity comparable to that of *B. thuringiensis* subsp. *israelensis* against second-instar *Aedes aegypti*. This indicated the presence of a novel mosquitocidal *cry* gene(s).

In order to clone the novel mosquitocidal *cry* gene(s), PCR was performed using a forward primer, 288-5 (5'-ACAAATTATAAGATTGGCT-3'), and two reverse primers, 288-31 (5'-ATCCCCTCCTGTATGACCGTCC-3') and 288-32 (5'-GAGTAATTGGCAGAAATTC-3'), which were designed on the basis of common DNA sequences of known mosquitocidal crystal proteins. The 1.6-kb and 1.8-kb PCR products were amplified by using 288-31 and 288-32 as reverse primers, respectively. We designated 1.6-kb and 1.8-kb PCR products as 288A and 288B, respectively. 288A and 288B were labeled and used as probes to hybridize with the total DNA obtained from *B. thuringiensis* subsp. *entomocidus* INA288. An XbaI fragment of about 8 kb was hybridized with the 288A probe. The 7- to 9-kb XbaI fragments were purified and cloned into the XbaI site of pUC119, and *Escherichia coli* DH5 α was transformed using these plasmids. Colony hybridization was performed for

screening against recombinant *E. coli* DH5 α colonies, and the plasmid was extracted from the positive clone. The positive clone contained an XbaI DNA insert of about 8 kb. Sequencing analysis revealed the presence of two open reading frames (ORFs) oriented in the same direction (Fig. 1). The first ORF corresponded to a polypeptide of 686 amino acids (aa) with a deduced molecular mass of 77.5 kDa. This protein was further classified as Cry44Aa1. The second ORF, located downstream of *cry44Aa1*, encoded a polypeptide of 541 aa with a deduced molecular mass of 64.1 kDa and was designated as ORF2-44A. A nucleotide sequence similar to the -35 and -10 regions of the σ^{35} -dependent promoter was found upstream of *cry44Aa*. A palindromic sequence that could form an mRNA hairpin loop with a ΔG of -20.53 kcal/mol and a shorter sequence that could act as a ρ -factor-independent transcriptional terminator were found downstream of the *orf2-44A* gene. Nucleotide sequences capable of acting as ribosome binding sites were also found just upstream of *cry44Aa* and *orf2-44A* (data not shown). These results indicate that *cry44Aa* and *orf2-44A* form an operon.

Labeled 288B was hybridized with a BamHI fragment of approximately 20 kb, and a lambda library of BamHI fragments of *B. thuringiensis* subsp. *entomocidus* INA288 DNA was constructed. The phage DNA was extracted from a positive lambda clone (lambda 288B) and was digested with various restriction enzymes. Restriction enzyme-digested DNA fragments were electrophoresed and Southern blotted to screen for an insert of a suitable size. The 7-kb BamHI-HindIII fragments and 8-kb HindIII fragments, which hybridized with the 288B probe, were cloned into pUC119 to yield pUC288B1 and pUC288B2. A partial nucleotide sequence of pUC288B1 and pUC288B2 in the region containing the *cry* gene was determined. Two ORFs that were oriented in the same direction were detected. The first ORF corresponded to a polypeptide of 683 aa with a deduced molecular mass of 77.4 kDa. This protein was further classified as Cry30Ba1. The second ORF, located downstream of *cry30Ba1* and coding for a polypeptide of 545 aa with a deduced molecular mass of 62.0 kDa, was designated ORF2-30B. There is no known Cry protein that shows high homology to Cry44Aa; the protein that showed maximum homology was Cry19Ba from *Bacillus thuringiensis* subsp. *higo* (32% identity). Therefore, this protein belongs to a

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FIG. 1. Organization of the genes in the *cry30Ba* and *cry44Aa* operons. The positions and directions of the genes are indicated by open arrows. The eight regions conserved among Cry proteins are indicated by black rectangles. The arrowhead and asterisks indicate the putative promoter region and the transcriptional terminator, respectively. Black diamonds indicate tentative ribosome binding sites.

novel class of δ endotoxins. Cry30Ba was similar to Cry30Aa from *Bacillus thuringiensis* subsp. *medellin* (58% identity). Both Cry44Aa and Cry30Ba contained the five conserved blocks (block 1 to block 5) that are present in almost all Cry proteins. On the other hand, ORF2-44A and ORF2-30B contained three conserved blocks (block 6 to block 8) that were present in the carboxyl-terminal half of 130-kDa-type crystal proteins, and the two ORFs showed high homology to those carboxyl-terminal halves (Fig. 1). Thus, *cry44Aa/orf2-44A* and *cry30Ba/orf2-30B* have a gene structure similar to that of the 130-kDa-type crystal protein genes. A similar gene structure, in which a typical 130-kDa protein *cry* gene was split into two separate ORFs, has been observed in *cry39A* (7), *cry40A* (GenBank

FIG. 2. Scanning electron microscopy showing spores and crystal-line inclusions produced by the *B. thuringiensis* parental strain and transformants. Magnification, $\times 10,000$.

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of crystal proteins produced by the *B. thuringiensis* parental strain and transformants. Lane 1, molecular mass markers.

accession no. AB074414), *cry40B* (GenBank accession no. AB112346), *cry10A* (13), and *cry19A* (12). The cause of the 130-kDa-type Cry protein's division into two segments has not been elucidated. At present, this gene configuration has been detected only in *cry* genes from mosquito strains.

In order to test the insecticidal activities of Cry44A and Cry30B, expression plasmids were constructed and introduced into an acrySTALLIFEROUS *Bacillus thuringiensis* subsp. *kurstaki* HD-1 mutant (Bt51) (15). When the *cry44Aa* and *cry30Ba* were expressed without their *orf2* genes, the resulting Bt51 transformants did not produce any detectable crystalline inclusion bodies (data not shown). Similar results have been reported previously (7, 12). As mentioned above, ORF2-44A and ORF2-30B correspond to the carboxyl-terminal half of a typical 130-kDa Cry protein. The 130-kDa Cry proteins with truncated carboxyl-terminal portions do not form crystalline inclusion bodies (1, 11, 14), and the carboxyl-terminal half of the 130-kDa Cry protein is considered to be involved in crystallization and/or stabilization but not in toxicity (10). Therefore,

TABLE 1. Mosquitocidal activities of purified crystals from *B. thuringiensis* strains

<i>B. thuringiensis</i> strain	Crystal composition	LC ₅₀ (μg/ml after 24 h) ^a		
		<i>Culex pipiens pallens</i>	<i>Anopheles stephensi</i>	<i>Aedes aegypti</i>
<i>B. thuringiensis</i> subsp. <i>entomocidus</i> INA288	Wild type	0.024 (0.019–0.030) ^c	6.91 (4.07–13.43)	0.035 (0.022–0.064)
Bt51-pHY44A	Cry44Aa and ORF2-44A	0.006 (0.004–0.008)	1.265 (0.357–1.790)	0.012 (0.008–0.017)
Bt51-pHY30B	Cry30Ba and ORF2-30B	>100 ^b	>100 ^b	>100 ^b
Bt51-pHY/IAaP-IVA	Cry4Aa	0.293 (0.119–0.831)	0.521 (0.341–0.748)	0.264 (0.097–0.455)

^a The crystals were assayed against first-instar larvae of *A. stephensi* and second-instar larvae of *C. pipiens* and *A. aegypti*.

^b At this concentration, no mortality was obtained.

^c Numbers in parentheses are 95% confidence limits, as determined by log probit analysis.

ORF2-44A and ORF2-30B were considered to have similar functions. When *cry44Aa* and *cry30Ba* were expressed with their *orf2* genes, recombinants Bt51-pHY44A and Bt51-pHY30B produced crystals that were detectable under a light microscope. Bt51 transformants and *B. thuringiensis* subsp. *entomocidus* INA288 were induced to sporulate and autolyse after 72 h of incubation. After being harvested and washed, spores and crystals were observed using a scanning electron microscope as described by Iizuka et al. (6). In comparison with wild-type INA288, which produced large cuboidal crystals (Fig. 2A), recombinants Bt51-pHY44A and Bt51-pHY30B produced smaller, amorphous crystals (Fig. 2B and C). These crystals were purified on discontinuous sucrose gradients as described by Nishimoto et al. (9) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). The molecular masses of major polypeptides in the crystals purified from wild-type INA288 were 74, 70, 65, 60, 43, 24, and 14 kDa. Two major polypeptides of 73 and 65 kDa were detected in crystals obtained from Bt51-pHY44A. The sequences of the first five N-terminal amino acids of 73- and 65-kDa polypeptides were identical to those of Cry44A and ORF2-44A, respectively. In the crystals obtained from Bt51-pHY30B, major polypeptides of 73 and 66 kDa were detected. The sequences of the first five N-terminal amino acids of 73- and 66-kDa polypeptides were identical to those of Cry30B and ORF2-30B, respectively.

Purified crystals obtained from Bt51 recombinants including Bt51-pHY/IAaP-IVA, which produced Cry4A crystals (4), and crystals obtained from *B. thuringiensis* subsp. *entomocidus* INA288 were tested against first-instar larvae of *Anopheles stephensi* and second-instar larvae of *Culex pipiens pallens* and *Aedes aegypti* (Table 1). Five larvae were transferred to each well of a 24-well titer plate that contained 950 μl of tap water per well. Fifty microliters of the serial dilutions of purified crystal proteins was added. Larval mortality was recorded after 24 h of incubation, and 50% mortality concentrations (LC₅₀s) were calculated by probit analysis. Thirty larvae were tested against each dilution, and the bioassays were performed more than three times. The Cry44A crystals showed strong toxicity against *C. pipiens* (LC₅₀ = 0.006 μg/ml) and *A. aegypti* (LC₅₀ = 0.012 μg/ml), and the Cry44A crystals were approximately 49 times and 22 times more toxic than Cry4A crystals against *C. pipiens* and *A. aegypti*, respectively. In addition, the Cry44A crystals were slightly toxic against *A. stephensi* (LC₅₀ = 1.265 μg/ml). Crystals from *B. thuringiensis* subsp. *entomocidus* INA288 showed similar tendencies in mosquitocidal activity. This suggests that Cry44Aa plays a major role in the mosqui-

cidal activity of *B. thuringiensis* subsp. *entomocidus* INA288 crystals. The investigation of the activation processes of Cry44Aa protoxins and binding properties of Cry44Aa toxins for brush border membranes prepared from mosquito larvae is in progress in order to gain an understanding of the mechanism of action of mosquitocidal crystal protein Cry44A. In contrast to Cry44A, the Cry30B crystals at a 100-μg/ml concentration were not toxic against any mosquito species tested in this study. Cry29Aa from *B. thuringiensis* subsp. *medellin* strain 161-131 is nontoxic to *C. pipiens*, *A. stephensi*, and *A. aegypti*. However, Cry29A shows a fourfold synergistic activity with Cry11Bb against *A. aegypti* (8). Therefore, Cry30Ba may also possess a similar synergistic property. Even if there is a synergy, it should be subtle and/or masked by the presence of other proteins in the native INA288 crystal since the native INA288 crystals, which contain both Cry44A and Cry30B, are four- or fivefold less toxic than pure Cry44A crystals. An additional bioassay in combination with other Cry proteins such as Cry44Aa is required.

In this paper, we reported the cloning and expression of two novel crystal protein genes, *cry44Aa/orf2-44A* and *cry30Ba/orf2-30B*, from highly mosquitocidal *B. thuringiensis* subsp. *entomocidus* INA288. The *cry44Aa/orf2-44A* gene is highly toxic to *C. pipiens* and *A. aegypti* and appears to be a promising alternative to *B. thuringiensis* subsp. *israelensis* or may be used in combination with *B. thuringiensis* subsp. *israelensis* toxins. It is essential to perform additional bioassays with these Cry toxins against the resistant mosquito colonies selected with *B. thuringiensis* subsp. *israelensis* toxins. These studies will serve as evidence that novel *cry* genes from INA288 such as *cry44Aa/orf2-44A* may be useful in managing resistance and/or as a component of synthetic combinations of mosquitocidal toxins.

Nucleotide sequence accession numbers. The nucleotide sequence data are available in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers AB161456 (*cry44Aa1* and *orf2-44A*) and AB125059 (*cry30Ba1* and *orf2-30B*).

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