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Identification of a Gene for Cyt1A-Like Hemolysin from *Bacillus thuringiensis* subsp. *medellin* and Expression in a Crystal-Negative *B. thuringiensis* Strain

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A gene designated *cyt1Ab1*, encoding a 27,490-Da protein, was isolated from *Bacillus thuringiensis* subsp. *medellin* (H30 serotype) by using an oligonucleotide probe corresponding to the *cyt1Aa1* gene. The sequence of the Cyt1Ab1 protein, as deduced from the sequence of the *cyt1Ab1* gene, was 86% identical to that of the Cyt1Aa1 protein and 32% identical to that of the Cyt2Aa1 protein from *B. thuringiensis* subsp. *kyushuensis*. The *cyt1Ab1* gene was flanked upstream by a *p21* gene, in the same orientation, encoding a 21,370-Da protein that showed 84% similarity to the putative chaperone P20 protein from *B. thuringiensis* subsp. *israelensis* and downstream, on the opposite strand, by a sequence showing 85% identity to the IS*240*A insertion sequence. The *cyt1Ab1* gene was expressed at a high level in a nontoxic strain of *B. thuringiensis* subsp. *israelensis* in which large inclusions of the Cyt1Ab1 protein were produced. Purified Cyt1Ab1 crystals were as hemolytic as those of the Cyt1Aa1 protein and were twice as hemolytic as those from the wild-type strain. Mosquitocidal activity toward *Aedes aegypti*, *Anopheles stephensi*, and *Culex pipiens* larvae was assayed. The toxicity of the Cyt1Ab1 protein was slightly lower than that of the Cyt1Aa1 protein for all three mosquito species, and Cyt1Ab1 was 150, 300, and 800 times less active toward *Culex*, *Anopheles*, and *Aedes* larvae, respectively, than were the native crystals from *B. thuringiensis* subsp. *medellin*.

Bacillus thuringiensis subsp. *israelensis* is the only bacterium other than *Bacillus sphaericus* that is currently used for mosquito and blackfly control. Both bacteria synthesize crystalline protein inclusions during sporulation that are toxic for the larvae upon ingestion. *B. thuringiensis* subsp. *israelensis* inclusions contain four major polypeptides, which in the new nomenclature are Cry4A (125 kDa), Cry4B (135 kDa), Cry11A (68 kDa), and Cyt1Aa (28 kDa) (9). The genes encoding *B. thuringiensis* subsp. *israelensis* Cry and Cyt toxins are located on a 72-MDa resident plasmid (19) and have been cloned and expressed in various hosts. Expression of *B. thuringiensis* subsp. *israelensis* genes, independently or in combination, in crystal-negative strains has led to determination of the specificities of each polypeptide for mosquito species and their synergistic interactions (2, 15, 25, 36). CytAa is a cytolysin and is poorly toxic. However, it acts in synergy with the other toxins (14, 36). Unlike Cry toxins, which are specific to insect larvae, Cyt toxins are cytolytic to various cells in vitro, including mammalian cells (8, 30). A variety of different Cyt toxins have been found in mosquitocidal strains, and the genes for most of them have been identified and sequenced; they are Cyt1A from *B. thuringiensis* subsp. *israelensis* (Cyt1Aa1) and *B. thuringiensis* subsp. *morrisoni* strain PG14 (Cyt1Aa2) (18, 33) and CytB (Cyt2Aa1) from the *B. thuringiensis* subsp. *kyushuensis* type strain (20), which is also found in *B. thuringiensis* subsp. *darmstadiensis* 73-E10-2 (17). The gene encoding the CytC toxin in *B. thuringiensis* subsp. *fukuokaensis* has not been isolated and sequenced (37). The two Cyt1Aa sequences differ only by one residue, whereas Cyt2Aa1 shares only 39% identity and 70% similarity with Cyt1Aa1 (20).

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There is continuous research toward identification of novel mosquitocidal toxins suitable for use if insects become resistant to existing toxins. *B. thuringiensis* subsp. *medellin* (H30 serotype) was reported in 1992 (24) and shows high mosquitocidal activity (23), specially toward *Anopheles* larvae, on which it is as toxic as *B. thuringiensis* subsp. *israelensis*. It is thus a potential candidate for use in mosquito control. Its crystalline proteins, which are different from those of *B. thuringiensis* subsp. *israelensis*, include major polypeptides of 100, 80, 75, 67, 65, 41 to 40, 30, and 28 kDa. Only the 30-kDa protein and, more weakly, the 28-kDa protein are immunologically related to *B. thuringiensis* subsp. *israelensis* toxins (23). The presence of a Cyt1A-like protein was confirmed by hybridization experiments using the *cyt1Aa1* gene as a probe on total DNA (26). Cyt1Aa proteins in other strains potentiate the activity of the other toxins. The contribution of this polypeptide in *B. thuringiensis* subsp. *medellin* to the overall toxicity is thus of interest, partly to envisage the possibility of combining the different toxic components in a single organism.

We isolated from *B. thuringiensis* subsp. *medellin* and sequenced a gene encoding a Cyt1A-like protein. We describe here the genetic environment of this cytolysin and the hemolytic and mosquitocidal activities of a *B. thuringiensis* crystal-negative strain transformed with the gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. thuringiensis* subsp. *medellin* 163-131 (H30 serotype) came from the IEBC Collection of the Unité des Bactéries Entomopathogènes, Institut Pasteur, Paris, France, and was used as a source of wild-type crystals and total DNA. *B. thuringiensis* subsp. *israelensis* 4Q2-72 carrying only the 72-MDa plasmid (a gift of D. Dean, Ohio State University, Columbus) and the recombinant strain *B. thuringiensis* 4Q7(pWF45), carrying the *cyt1Aa1* gene cloned from *B. thuringiensis* subsp. *israelensis* (36), were used as controls for hemolytic and mosquitocidal activities and for purification of crystalline inclusions.

Escherichia coli TG1 (K-12 Δ(*lac-proAB*) *supE thi hsdD* F′(*raD36 proA*$^+$ *proB*$^+$ *lacI lac* ZΔM15) was used as the cloning host. Transformation was performed as

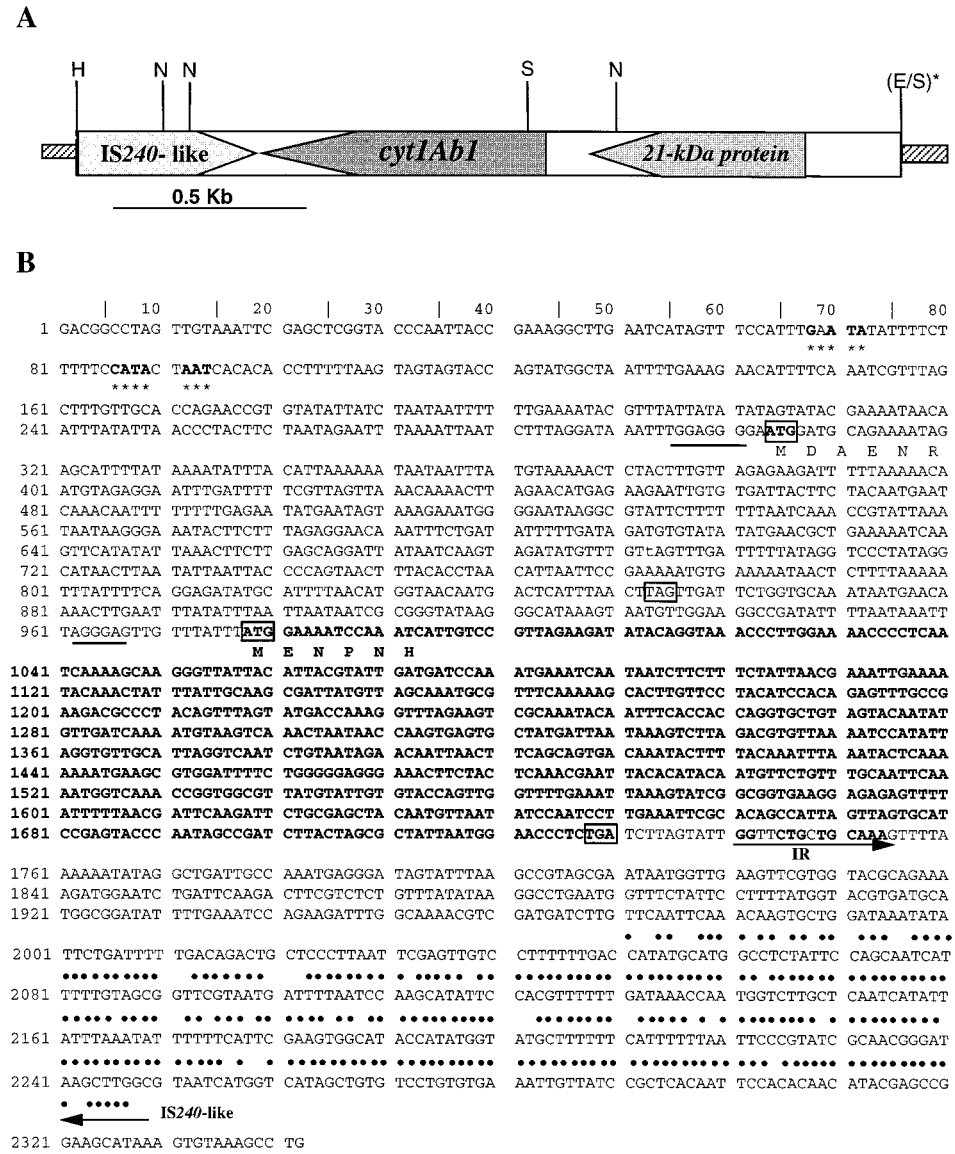

FIG. 1. Organization of the fragment pCytM. (A) Restriction map of the recombinant plasmid pCytM containing the *cyt1Ab1* gene. The positions and directions of transcription of the *cyt1Ab1* and IS*240*-like genes and the gene coding for the 21-kDa protein are represented by arrows. The hatched bars represent the pHT315 vector. Abbreviations: H, *Hin*dIII; N, *Nsi*I; S, *Sty*I; E, *Eco*RI; S, *Sma*I. The asterisk indicates a site that was lost during cloning experiments. (B) Nucleotide sequence of the *cyt1Ab1* gene and flanking sequences. Boldfaced characters represent the *cyt1Ab1* gene nucleotides. The start codons are boxed. Potential ribosome sites are underlined. The putative promoter region is labeled with asterisks. The nucleotides with dots below them are identical to those of IS*240*A. The consensus portions of the inverted repeat (IR) are underlined with arrows.

previously described (21) on Luria-Bertani plates containing ampicillin (100 μg per ml). The crystal-negative strain *B. thuringiensis* subsp. *thuringiensis* SPL407 (H1 serotype) was used as a recipient for transformation by electroporation (22). Erythromycin was used at 25 μg per ml for bacterial selection.

The shuttle vector pHT315 (3) was used for cloning experiments. The recombinant plasmid pCB4 was the source of the *cyt1Aa1* gene from *B. thuringiensis* subsp. *israelensis* (5).

DNA manipulations. All restriction enzymes were used as recommended by the manufacturers. Protocols for restriction endonuclease digestions and ligation were carried out as previously described by Sambrook et al. (28).

Total DNA from *B. thuringiensis* subsp. *medellin* cells was isolated as described by Delécluse et al. (14). Plasmid DNA was extracted from *E. coli* by a standard alkaline lysis procedure (4) and further purified by using the Qiagen plasmid kit. DNA fragments were analyzed by electrophoresis on 0.7 or 1.2% agarose gels and were, when necessary, eluted from agarose gels by using the Prep-A-Gene DNA purification matrix kit (Bio-Rad, Hercules, Calif.). Hybridization experiments were performed on Hybond N+ filters (Amersham Life Science). Labeling was performed with peroxidase by using the ECL direct nucleic acid labeling system (Amersham).

B. thuringiensis subsp. *medellin* DNA was hydrolyzed by various restriction enzymes and tested for hybridization with the 1.7-kb *Bam*HI-*Eco*RI fragment isolated from pCB4 carrying the *cyt1Aa1* gene. Three fragments (the 7-kb *Eco*RI, 2.9-kb *Eco*RI-*Eco*RV, and 2.2-kb *Hin*dIII-*Eco*RV fragments) hybridized with the probe. Size-selected (2- to 3-kb) *Hin*dIII-*Eco*RV fragments were inserted into the *Hin*dIII-*Sma*I site of the shuttle vector pHT315. *E. coli* TG1 transformants were tested for hybridization with the labeled probe. The plasmid from one clone (pCytM) was selected and used for further experiments. It contained a 2.2-kb insert, for which a restriction map was established (Fig. 1A).

Nucleotide sequencing was performed on alkali-denatured plasmids by using the Sequenase Quick sequencing kit, version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) and [α-^{35}S]dATP (>37 TBq/mmol; Amersham) according to the method of Sanger et al. (29). Synthetic oligonucleotides prepared by Eurogentec or Institut Pasteur were used for the sequencing of both strands by the gene walking technique.

The nucleotide sequence of the gene was compared with data from GenBank-EMBL, and the corresponding amino acid sequence was compared with sequences from the Swiss-Prot data banks. Alignment and comparisons were performed with the Genetics Computer Group package.

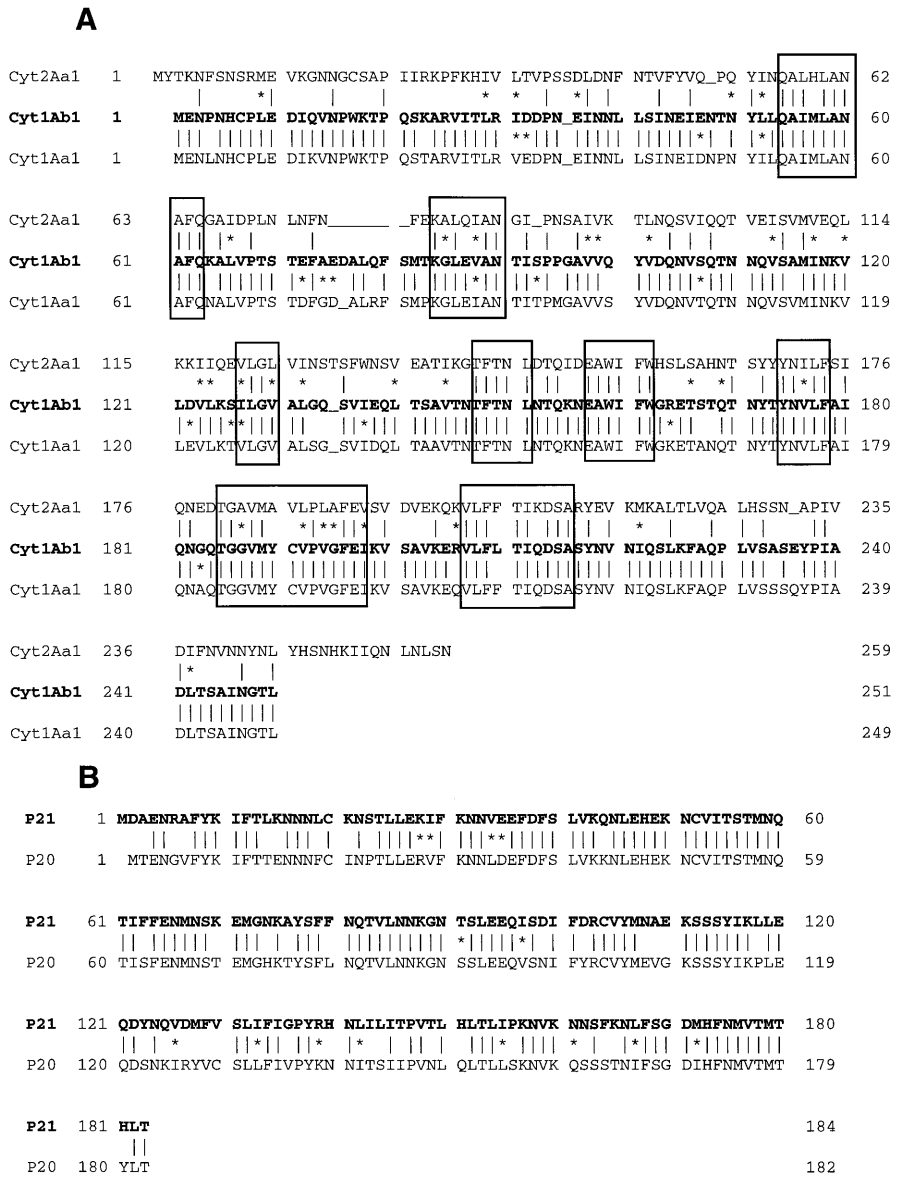

FIG. 2. Sequences of the Cyt1Ab1 and P21 proteins. (A) Comparison of the sequence of Cyt1Ab1 with the Cyt1Aa1 and Cyt2Aa1 protein sequences. Identical amino acids are linked by lines; (B) comparison of P21 from *B. thuringiensis* subsp. *medellin* and P20 from *B. thuringiensis* subsp. *israelensis* protein sequences. Residues which are functionally similar are indicated with asterisks (accepted conservative replacements are I, L, V, and M; D and E; Q and N; K and R; T and S; G and A; and F and Y). The conserved blocks are boxed.

Protein analysis. *B. thuringiensis* cells were grown in UG medium (10), supplemented with erythromycin when necessary, on an orbital shaker at 30°C until cell lysis occurred. Spores and crystals were harvested, washed once with 1 M NaCl and twice with distilled water containing 1 mM phenylmethylsulfonyl fluoride and 10 mM EDTA, and resuspended in the same solution. Crystals were separated from spores on a discontinuous sucrose gradient (79 to 67%) by using an SW28 swinging-bucket rotor in a Beckman L8-55 ultracentrifuge at 25,000 rpm and 4°C for 16 h. Protein concentrations of the alkali-solubilized crystal preparations were measured by the Bradford assay with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described by Delécluse et al. (12). Proteins were transferred onto a Hybond-C super membrane (Amersham) and detected immunologically by using the ECL Western blotting system kit (Amersham) as recommended by the manufacturer. Antisera directed against either *B. thuringiensis* subsp. *israelensis* purified Cyt1Aa1 toxin or *B. thuringiensis* subsp. *medellin* total purified crystals were used to detect the different produced proteins.

Mosquitocidal activity assay. Larvae of *Culex pipiens pipiens* (strain Montpellier), *Anopheles stephensi* (strain ST15), and *Aedes aegypti* (strain Bora-Bora) were reared in dechlorinated water in the laboratory at 25°C with a 14-h/10-h day/night photoperiod. Purified crystals were added to 10 ml of demineralized water in petri dishes (diameter, 5.5 cm) and tested in duplicate against 20 young fourth-instar larvae of *Aedes aegypti* and *C. pipiens* and third-instar larvae of *Anopheles stephensi*. At least five concentrations of crystals were tested. Each experiment was run at least three times. Larval mortality was recorded after 24 h, and 50% lethal concentration (LC_{50}) and LC_{90} values were determined by probit analysis on a Macintosh computer (Apple Computer, Paris, France). LC values are expressed as means ± standard error (SE).

Hemolytic activity assay. Ten-milliliter volumes of sheep and horse erythrocytes (Sanofi Diagnostic Pasteur) were each washed twice in 0.015 M Tris-HCl (pH 8)–0.17 M NaCl and resuspended to 2.25% (6×10^8/ml) in the same buffer. A sample (0.5 ml) of this solution was added to 1 ml of serially diluted, alkali-solubilized crystals. These dilutions were incubated at 37°C for 45 min and then centrifuged at 2,500 rpm (MSE Microcentaur; Serlabo, Paris, France) for 2 min. The amount of hemoglobin released was estimated by measuring the absorbance of the supernatant at 540 nm. The 50% hemolytic dose (HD_{50}) was defined as the amount of protein needed to release half the hemoglobin from the erythrocytes (optical density at 540 nm, 0.5) and was estimated by a log-probit program. Each assay was run two or three times.

Nucleotide sequence accession number. The nucleotide sequence data are available in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers X98793 (*cry1Ab1*) and X98794 (*p21* gene).

RESULTS

Cloning and sequence analysis. A *B. thuringiensis* subsp. *medellin* DNA library was established in *E. coli* (see Materials and Methods) and screened for hybridization. One positive clone was selected with the *cyt1Aa1* gene probe from plasmid pCB4, and the corresponding plasmid, pCytM, was used for further experiments. It contained a 2.2-kb insert (Fig. 1A). The sequence of the whole fragment was determined on both strands, and the positions and the direction of transcription of the genes were determined (Fig. 1). The pCytM fragment contained a gene with an open reading frame (ORF) encoding a polypeptide of 251 amino acids with a predicted molecular mass of 27,490 Da. This protein was designated Cyt1Ab1 by the Cry gene nomenclature committee (9a). Ten nucleotides upstream from the start codon was a putative ribosome binding site (AGGGAGT) with a ΔG of -30.9 kJ/mol, calculated according to the method of Tinoco et al. (31). The amino acid sequence of the protein encoded by the gene fragment was compared to those of Cyt1A toxins from *B. thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *morrisoni* in the Swiss-Prot data bank as well as that of the Cyt2Aa1 toxin from *B. thuringiensis* subsp. *kyushuensis*. It was similar to that of the Cyt1Aa1 toxin (86% identity) but only 32% identical to that of Cyt2Aa1 from *B. thuringiensis* subsp. *kyushuensis* (Fig. 2A).

Eleven nucleotides downstream from the stop codon of the *cyt1Ab1* gene was an insertion sequence very similar to IS*240*A in the direction opposite the *cyt1Ab1* gene (Fig. 1B). It included an inverted repeat conforming to the IS*240* consensus sequence (13). The 225 nucleotides adjacent to the inverted repeat were 85% identical to the IS*240*A gene, but no ORF was found. Ending 125 nucleotides upstream from the *cyt1Ab1* ATG was another ORF in the same orientation as the *cyt1Ab1* gene. This ORF encoded a polypeptide of 184 amino acids with a predicted molecular mass of 21,370 Da. This protein, named P21, showed 84% similarity to the P20 protein found by Adams et al. (1) (Fig. 2B). No promoter was found between *p21* and *cyt1Ab1*, but 237 nucleotides upstream from the ATG of the *p21* gene is a sequence similar to the consensus sequence of the σ^E recognition site of *B. thuringiensis* (6, 7, 16).

Expression of Cyt1Ab1 in a crystal-negative strain of *B. thuringiensis*. To determine the toxicity of cytolysin Cyt1Ab1, *B. thuringiensis* SPL407 was transformed with the plasmid pCytM by electroporation. SPL407(pCytM) recombinant cells sporulated and produced large parasporal inclusions. No inclusions were visible in the control, *B. thuringiensis* SPL407 containing the vector pHT315 alone. Inclusions from *B. thuringiensis* SPL407(pCytM) were purified and compared with those produced by *B. thuringiensis* 4Q7(pWF45), carrying the *cyt1Aa1* gene, *B. thuringiensis* subsp. *israelensis* 4Q2-72, and *B. thuringiensis* subsp. *medellin* 163-131 by SDS-PAGE (Fig. 3A). The recombinant *B. thuringiensis* SPL407(pCytM) displayed a major polypeptide of 30 kDa and minor bands of ca. 25 and 20 kDa. The recombinant *B. thuringiensis* 4Q7(pWF45) produced a 28-kDa protein. Immunorelationships were investigated with antisera directed against either *B. thuringiensis* subsp. *israelensis* Cyt1Aa1 protein or total crystals from *B. thuringiensis* subsp. *medellin* 163-131 (Fig. 3B). Both *B. thuringiensis* SPL407 (pCytM) and *B. thuringiensis* 4Q7(pWF45) cross-reacted strongly with the anti-Cyt1Aa1 serum (Fig. 3B). Serum against *B. thuringiensis* subsp. *medellin* total crystals strongly labeled a doublet of 30 and 28 kDa in *B. thuringiensis* SPL407(pCytM) and a doublet of 28 and 25 kDa in *B. thuringiensis* 4Q7(pWF45) and weakly labeled proteins of 20 and 50 kDa in *B. thuringiensis* SPL407(pCytM) and of 65 kDa in *B. thuringiensis* 4Q7 (pWF45) (Fig. 3B). Antiserum against *B. thuringiensis* subsp. *medellin* total crystals cross-reacted with the doublet of 30 and 28 kDa and the 94-kDa protein of the total *B. thuringiensis* subsp. *medellin* crystals but reacted only with the 28-kDa protein from total crystals of *B. thuringiensis* subsp. *israelensis* (data not shown).

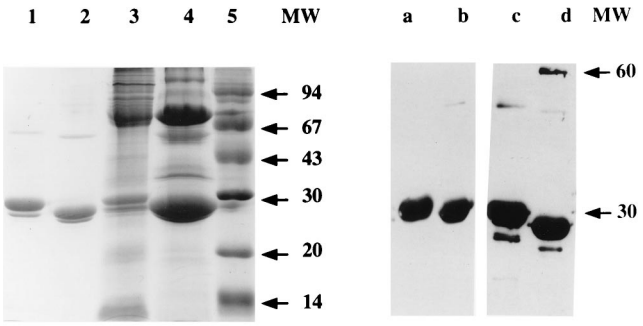

FIG. 3. Protein analysis of purified Cyt1Ab1 inclusions. (A) Purified inclusions were subjected to electrophoresis in SDS–12% polyacrylamide gels and stained with Coomassie brilliant blue. Lane 1, 5 μg of Cyt1Ab1 recombinant protein; lane 2, 5 μg of Cyt1Aa1 recombinant protein; lane 3, 25 μg of *B. thuringiensis* subsp. *medellin* total crystals; lane 4, 25 μg of *B. thuringiensis* subsp. *israelensis* 4Q2-72 total crystals; lane 5, low-molecular-weight markers from Pharmacia. (B) Western blot of 5 μg of purified Cyt1Ab1 (lanes a and c) and 5 μg of Cyt1Aa1 (lanes b and d) inclusions. The filter was incubated with antiserum (diluted 1/2,000) raised against solubilized Cyt1Aa1 inclusions (lanes a and b) or with antiserum (1/5,000) raised against solubilized total crystals from *B. thuringiensis* subsp. *medellin* (lanes c and d).

Toxic activity of the Cyt1Ab1 protein inclusions. Solubilized crystals of *B. thuringiensis* SPL407(pCytM), *B. thuringiensis* 4Q7(pWF45), *B. thuringiensis* subsp. *israelensis* 4Q2 72 and *B. thuringiensis* subsp. *medellin* were assayed for hemolytic activity on sheep and horse erythrocytes. The cloned Cyt1Ab1 protein, the Cyt1Aa1 protein alone, and *B. thuringiensis* subsp. *israelensis* total crystals (Table 1) all had similar HD_{50} values on sheep erythrocytes of about 1 μg of solubilized protein. *B. thuringiensis* subsp. *medellin* crystals were less hemolytic. Horse erythrocytes were at least six times less susceptible to these hemolysins than sheep cells. *B. thuringiensis* SPL407(pCytM) and *B. thuringiensis* subsp. *medellin* were specifically less hemolytic to horse cells than *B. thuringiensis* subsp. *israelensis* cytotoxins. Purified inclusions from *B. thuringiensis* SPL407(pCytM), *B. thuringiensis* 4Q7(pWF45), *B. thuringiensis* subsp. *israelensis* 4Q2 72, and *B. thuringiensis* subsp. *medellin* were assayed for mosquitocidal activity on larvae of *Aedes aegypti*, *C. pipiens*, and *Anopheles stephensi*. Purified Cyt1Ab1 crystals were larvicidal toward *C. pipiens*, *Anopheles stephensi*, and *Aedes aegypti*, with LC_{50} values of approximately 5, 20, and 58 μg of protein/ml, respectively, after 48 h of exposure (Table 2). They were 3 to 50 times less toxic than Cyt1Aa1 crystals from *B. thuringiensis* 4Q7(pWF45) with a different relative specificity for *Aedes* and *Anopheles* larvae. Purified Cyt1Aa1 and Cyt1Ab1 crystals from both recombinant strains were at least 200 times less larvicidal than the crystals from the respective wild-type strains.

DISCUSSION

We report the cloning and characterization of a gene encoding a 27,490-Da cytolysin from the mosquitocidal strain *B.*

TABLE 1. Comparison of the hemolytic activities of recombinant and wild-type strains

Strain	Hemolytic activity on erythrocytes from[a]:			
	Sheep		Horses	
	HD_{50}	HD_{90}	HD_{50}	HD_{90}
B. thuringiensis subsp. *israelensis* 4Q2 72	1.1 ± 0.5	13.4 ± 12.9	7.5 ± 3.6	220.0 ± 136.0
B. thuringiensis subsp. *medellin* 163-131	4.7 ± 0.1	12.2 ± 1.3	70.1 ± 13.0[b]	557.5 ± 254.5
B. thuringiensis subsp. *israelensis* 4Q7(pWF45) (Cyt1Aa1)	0.5 ± 0.1[b]	0.9 ± 0.1	2.7 ± 1.0	11.0 ± 2.1
B. thuringiensis 407(pCytM) (Cyt1Ab1)	1.4 ± 0.4[b]	3.1 ± 0.9	32.2 ± 3.9	206.0 + 118.0

[a] Hemolytic doses are expressed in micrograms of protein. Unless otherwise noted, values are the means of data from three experiments ± SE.
[b] Values are means of data from two experiments ± SE.

thuringiensis subsp. *medellin* 163-131. This gene, *cyt1Ab1*, is very similar to the *cyt1Aa1* gene of *B. thuringiensis* subsp. *israelensis*, and the encoded protein, Cyt1Ab1, is also very similar to the Cyt1Aa1 protein. Moreover, some blocks appear to be conserved between the different Cyt proteins (Fig. 2A). The level of expression of the *cyt1Ab1* gene in a *B. thuringiensis* strain is very high, leading to the production of parasporal inclusions as large as those in the wild-type *B. thuringiensis* subsp. *medellin* and in *B. thuringiensis* 4Q7(pWF45), which produces the Cyt1Aa1 inclusions. Moreover, the hemolytic activities of the recombinant strains containing either Cyt1Aa1 or Cyt1Ab1 are very similar, whereas *B. thuringiensis* subsp. *medellin* total crystals are less hemolytic than *B. thuringiensis* subsp. *israelensis* total crystals. This discrepancy may be due to the amount of the cytolysin in the total crystals. Cyt1Aa1 protein represents about 50% of the total *B. thuringiensis* subsp. *israelensis* crystals (data not shown), whereas Cyt1Ab1 protein constitutes a much smaller proportion of *B. thuringiensis* subsp. *medellin* total crystals, as assessed by SDS-PAGE.

The two proteins have significantly different toxic specificities toward horse erythrocytes in vitro and on mosquito larvae in vivo. The differences in amino acid sequences of the Cyt1Ab1 and Cyt1Aa1 proteins may play a role in the toxicity. Ward et al. (34) showed that replacement of an amino acid (especially at position 78, 124, or 204) by alanine in the Cyt1Aa1 protein resulted in modification of the toxicity compared to that of the wild type. All residues recorded by Ward et al. (34) are found to be similar in the Cyt1Ab1 protein except the one located at position 78. The importance of the changes of other residues has to be tested in order to determine their influence on toxicity.

Despite the fact that the molecular weights of these two Cyt1A proteins, as deduced by their amino acid sequences, are the same, Cyt1Ab1 always migrated as a 30-kDa polypeptide whereas Cyt1Aa1 migrated as a 28-kDa polypeptide. This might be due to charge differences in amino acid residues or to conformational differences or glycosylation of the protein. Moreover, both Cyt1A proteins, in certain conditions visible on SDS-PAGE, can aggregate and form a dimer of ca 60 kDa (data not shown).

The organization of the environment of the *cyt1Ab1* gene encoding this protein is different from what was previously found in *B. thuringiensis* subsp. *israelensis*. Upstream from the cytolysin gene is a gene very similar to the one encoding the P20 protein in *B. thuringiensis* subsp. *israelensis* (16, 35). This gene, named *p21*, is transcribed in the same direction as the *cyt1Aa1* gene. Upstream from the *p21* gene is a putative promoter sequence similar to the consensus for the promoter σ^E, described by Dervyn et al. (16). Thus, these two genes may form an operon under the transcriptional control of σ^E.

In *B. thuringiensis* subsp. *israelensis*, the *cry11A* gene as well as those coding for the 19- and 20-kDa proteins map close together, are transcribed in the same direction, and are organized in an operon independent of and in the orientation opposite that of the *cytAa1* gene (16, 35). In *B. thuringiensis* subsp. *medellin*, the *p21* gene is close to the *cyt1Aa1* gene, is in the same orientation, and probably is transcribed from the same promoter (Fig. 1B). The deduced P21 protein is similar to the P20 protein of *B. thuringiensis* subsp. *israelensis*. In Cyt1Aa1, residues Arg-25 and Arg-30 have been shown to be essential for Cyt1Aa1 crystal formation in *E. coli* (34), and these residues are present in the Cyt1Ab1 protein. The P20 protein also promotes Cyt1Aa1 crystal formation in *B. thuringiensis* during sporulation (35). The presence of the P21 protein might protect Cyt1Ab1 from degradation, playing the role of a chaperone protein as P20 protein presumably does for Cyt1Aa1 (32). Thus, the presence of the *p21* gene in the same putative operon as the *cyt1Ab1* gene may result in a higher level of production of the corresponding protein.

Downstream from the *cyt1Ab1* gene is a sequence very similar to that of IS*240*A. No IS*240*A transposase gene is present within this sequence, but the inverted repeat is similar to the consensus sequence of IS*240*. The homology between this IS*240*A-like sequence from *B. thuringiensis* subsp. *medellin* and IS*240*A suggests that the former was probably an ancestral IS*240* which underwent some mutations. IS*240*A has previ-

TABLE 2. Mosquitocidal activity of purified crystals from *B. thuringiensis* strains

Crystals	LC units	Mosquitocidal activity on[a]:					
		Aedes aegypti		*G. pipiens*		*Anopheles stephensi*	
		LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
4Q2 72	ng/ml	2.0 ± 0.2	3.6 ± 0.4	2.5 ± 1.1	6.9 ± 2.9	0.8 ± 0.3	2.4 ± 0.5
163-131	ng/ml	65.3 ± 6.6	123.0 ± 12.3	38.0 ± 10.3	93.6 ± 33.5	58.4 ± 2.5	192.0 ± 23.3
4Q7(pWF45) (Cyt1Aa1)	μg/ml	0.88 ± 0.22	2.0 ± 1.0	1.2 ± 0.5	5.0 ± 0.9	6.3 ± 1.3	18.9 ± 3.7
407(pCytM) (Cyt1Ab1)	μg/ml	58.4 ± 9	239.7 ± 56	5.7 ± 3.1	29.7 ± 17.3	20.0 ± 4.9	114.0 ± 57.7

[a] Values are means of data from at least three experiments ± SE, obtained after 48 h of larval exposure.

ously been found close to toxin genes such as *cry4A* (11, 13) and in other *B. thuringiensis* mosquitocidal strains (27). IS*240* may be involved in the dispersion of these toxin genes.

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REFERENCES

1. **Adams, L. F., J. E. Visick, and H. R. Whiteley.** 1989. A 20-kilodalton protein is required for efficient production of the *Bacillus thuringiensis* subsp. *israelensis* 27-kilodalton crystal protein in *Escherichia coli*. J. Bacteriol. **171:** 521–530.
2. **Angsuthanasombat, C., N. Crickmore, and D. J. Ellar.** 1992. Comparison of *Bacillus thuringiensis* subsp. *israelensis* CryIVA and CryIVB cloned toxins reveals synergism in vivo. FEMS Microbiol. Lett. **94:**63–68.
3. **Arantes, O., and D. Lereclus.** 1991. Construction of cloning vectors for *Bacillus thuringiensis*. Gene **108:**115–119.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Bourgouin, C., A. Klier, and G. Rapoport.** 1986. Characterization of the genes encoding the haemolytic toxin and the mosquitocidal delta-endotoxin of *Bacillus thuringiensis israelensis*. Mol. Gen. Genet. **205:**390–397.
6. **Brown, K. L., and H. R. Whiteley.** 1988. Isolation of a *Bacillus thuringiensis* RNA polymerase capable of transcribing crystal protein genes. Proc. Natl. Acad. Sci. USA **85:**4166–4170.
7. **Brown, K. L., and H. R. Whiteley.** 1990. Isolation of the second *Bacillus thuringiensis* RNA polymerase that transcribes from a crystal protein gene promoter. J. Bacteriol. **172:**6682–6688.
8. **Chilcott, C. N., and D. J. Ellar.** 1988. Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *in vitro*. J. Gen. Microbiol. **134:**2551–2558.
9. **Crickmore, N., D. R. Zeigler, J. Feitelson, E. Schnepf, B. Lambert, D. Lereclus, C. Gawron-Burke, and D. H. Dean.** 1995. Revision of the nomenclature for the *Bacillus thuringiensis cry* genes, p. 14. *In* Abstracts of the Society for Invertebrate Pathology 28th Annual Meeting, Ithaca, N.Y., 16 to 21 July 1995.

9a. **Dean, D., and D. Zeigler.** Personal communication.

10. **de Barjac, H., and M.-M. Lecadet.** 1976. Dosage biochimique de l'exotoxine thermostable de *B. thuringiensis* d'après l'inhibition d'ARN-polymérases bactériennes. C. R. Acad. Sci. **282:**2119–2122.
11. **Delécluse, A., C. Bourgouin, A. Klier, and G. Rapoport.** 1989. Nucleotide sequence and characterization of a new insertion element, IS*240*, from *Bacillus thuringiensis israelensis*. Plasmid **21:**71–78.
12. **Delécluse, A., C. Bourgouin, A. Klier, and G. Rapoport.** 1988. Specificity of action on mosquito larvae of *Bacillus thuringiensis israelensis* toxins encoded by two different genes. Mol. Gen. Genet. **214:**42–47.
13. **Delécluse, A., C. Bourgouin, G. Menou, D. Lereclus, A. Klier, and G. Rapoport.** 1990. IS*240* associated with the *cryIVA* gene from *Bacillus thuringiensis israelensis* belongs to a family of Gram (+) and Gram (−) IS elements. *In* M. M. Zukowski, A. T. Ganesan, and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 3. Academic Press, Inc., San Diego, Calif.
14. **Delécluse, A., J.-F. Charles, A. Klier, and G. Rapoport.** 1991. Deletion by in vivo recombination shows that the 28-kilodalton cytolytic polypeptide from *Bacillus thuringiensis* subsp. *israelensis* is not essential for mosquitocidal activity. J. Bacteriol. **173:**3374–3381.
15. **Delécluse, A., S. Poncet, A. Klier, and G. Rapoport.** 1993. Expression of *cryIVA* and *cryIVB* genes, independently or in combination, in a crystal-negative strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3922–3927.
16. **Dervyn, E., S. Poncet, A. Klier, and G. Rapoport.** 1995. Transcriptional regulation of the *cryIVD* gene operon from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **177:**2283–2291.
17. **Drobniewski, F. A., and D. J. Ellar.** 1989. Purification and properties of a 28-kilodalton hemolytic and mosquitocidal protein toxin of *Bacillus thuringiensis* subsp. *darmstadiensis* 73-E10-2. J. Bacteriol. **171:**3060–3067.
18. **Earp, D. J., and D. J. Ellar.** 1987. *Bacillus thuringiensis* var. *morrisoni* strain PG14: nucleotide sequence of a gene encoding a 27 kDa crystal protein. Nucleic Acids Res. **15:**3619.
19. **González, J. M. J., and B. C. Carlton.** 1984. A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. Plasmid **11:**28–38.
20. **Koni, P. A., and D. J. Ellar.** 1993. Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. J. Mol. Biol. **229:**319–327.
21. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119:**1072–1074.
22. **Lereclus, D., O. Arantès, J. Chaufaux, and M.-M. Lecadet.** 1989. Transformation and expression of a cloned d-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol. Lett. **60:**211–218.
23. **Orduz, S., T. Diaz, I. Thiéry, J.-F. Charles, and W. Rojas.** 1994. Crystal proteins from *Bacillus thuringiensis* serovar *medellin*. Appl. Microbiol. Biotechnol. **40:**794–799.
24. **Orduz, S., W. Rojas, M. M. Correa, A. E. Montoya, and H. de Barjac.** 1992. A new serotype of *Bacillus thuringiensis* from Colombia toxic to mosquito larvae. J. Invertebr. Pathol. **59:**99–103.
25. **Poncet, S., A. Delécluse, A. Klier, and G. Rapoport.** 1995. Evaluation of synergistic interactions among the CryIVA, CryIVB and CryIVD toxic components of *Bacillus thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. **66:**131–135.
26. **Ragni, A., I. Thiéry, and A. Delécluse.** 1996. Characterization of six highly mosquitocidal *Bacillus thuringiensis* strains that do not belong to H-14 serotype. Curr. Microbiol. **32:**48–54.
27. **Rosso, M.-L., and A. Delécluse.** Distribution of the insertion sequence IS*240* among *B. thuringiensis* strains. Curr. Microbiol., in press.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Thomas, W. E., and D. J. Ellar.** 1983. Mechanism of action of *Bacillus thuringiensis* var. *israelensis* insecticidal δ-endotoxin. FEBS Lett. **154:**362–368.
31. **Tinoco, I. J., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla.** 1973. Improved estimation of secondary structure in ribonucleic acids. Nature (London) New Biol. **246:**40–41.
32. **Visick, J. E., and H. R. Whiteley.** 1991. Effect of a 20-kilodalton protein from *Bacillus thuringiensis* subsp. *israelensis* on the production of the CytA protein by *Escherichia coli*. J. Bacteriol. **173:**1748–1756.
33. **Waalwijk, C., A. M. Dullemans, M. E. S. vanWorkum, and B. Visser.** 1985. Molecular cloning and the nucleotide sequence of the M_r 28000 crystal protein gene of *Bacillus thuringiensis* subsp. *israelensis*. Nucleic Acids Res. **13:**8207–8217.
34. **Ward, E. S., D. J. Ellar, and C. N. Nicholls.** 1988. Single amino acid changes in the *Bacillus thuringiensis* var. *israelensis* δ-endotoxin affect the toxicity and expression of the protein. J. Mol. Biol. **202:**527–535.
35. **Wu, D., and B. A. Federici.** 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. J. Bacteriol. **175:**5276–5280.
36. **Wu, D., J. J. Johnson, and B. A. Federici.** 1994. Synergism of mosquitocidal toxicity between CytA and CryIVD proteins using inclusions produced from cloned genes of *Bacillus thuringiensis*. Mol. Microbiol. **13:**965–972.
37. **Yu, Y.-M., M. Ohba, and S. S. Gill.** 1991. Characterization of mosquitocidal activity of *Bacillus thuringiensis* subsp. *fukuokaensis* crystal proteins. Appl. Environ. Microbiol. **57:**1075–1081.