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Cryptic endotoxic nature of *Bacillus thuringiensis* Cry1Ab insecticidal crystal protein

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Abstract Cry1Ab is one of the most studied insecticidal proteins produced by *Bacillus thuringiensis* during sporulation. Structurally, this protoxin has been divided in two domains: the N-terminal toxin core and the C-terminal portion. Although many studies have addressed the biochemical characteristics of the active toxin that corresponds to the N-terminal portion, there are just few reports studying the importance of the C-terminal part of the protoxin. Herein, we show that Cry1Ab protoxin has a unique natural cryptic endotoxic property that is evident when their halves are expressed individually. This toxic effect of the separate protoxin domains was found against its original host *B. thuringiensis*, as well as to two other bacteria, *Escherichia coli* and *Agrobacterium tumefaciens*. Interestingly, either the fusion of the C-terminal portion with the insecticidal domain-III or the whole N-terminal region reduced or neutralized such a toxic effect, while a non-Cry1A peptide such as maltose binding protein did not neutralize the toxic effect. Furthermore, the C-terminal domain, in addition to being essential for crystal formation and solubility, plays a crucial role in neutralizing the toxicity caused by a separate expression of the insecticidal domain much like a dot/anti-dot system.

Keywords: Crystal toxin; Insecticide; Cry1A; Endotoxin; *Bacillus thuringiensis*

1. Introduction

Bacillus thuringiensis is a gram-positive soil bacterium that forms parasporal crystals during sporulation that are mainly composed of one of several insecticidal proteins highly specific for different insect larvae and nematodes [1,2]. These insecticidal toxins form a large family of out-inside ionic channels with more than a hundred members described so far [3,4]. The mode of action of *B. thuringiensis* toxin could be summarized in four steps: (i) crystal solubilization in the insect's midgut, (ii) proteolysis of the protoxins to yield toxins, (iii) binding of toxins to a receptor and then, ion channel formation on columnar cells, and (iv) osmotic dysfunction of the insect's intestinal epithelium [3,5].

The bipyramidal crystals, formed by Cry1A protoxins of 120–140 kDa, have been extensively studied during past decades. These bodies are soluble into high pH and produce ~70 kDa toxins upon trypsinization [6,7]. The 3-D structure of the Cry1Aa toxin showed a conserved three-domain configuration (domains I, II and III), which seems to be essential for the lytic activity [8]. Although the spatial structure of Cry1A protoxins is not yet determined, it has been divided in two portions: the N-terminal containing the insecticidal toxin and the C-terminal, whose function is still not well understood [9,10]. The N-terminal half of the protoxin is predominantly hydrophobic whereas the C-terminal half is hydrophilic. C-terminal halves of the Cry1A molecules contain 14 of the total 16 cysteines and 31 of the total 34 protoxin lysines. The thiol groups of the solubilized crystal protein are exposed on the surface of the molecule, and the disulfide bonds stabilize the crystal structure [11].

Interestingly, the C-terminal half of Cry1A primary structure is highly conserved among the related crystal proteins [12,13]. Whether this sequence conservation reflects an essential structural requirement for crystal formation and/or a requirement for correct folding of the N-terminal toxic moiety is unclear. Recently, reports have provided clues for a possible role of this domain in the protoxin's biological properties. For example, chimeric protoxins produced by exchange of C-terminal portions between Cry1Ab and Cry1C became more effective against *Spodoptera exigua* [14]. Other authors reported that mutations in the primary sequence of the C-terminal portion of the Cry1Ab protoxin could affect the solubility of inclusion bodies [15]. On the other hand, the replacement of one cysteine of the native Cry1Ab C-terminal by a heterogeneous sequence, affected the formation of crystals. The latter supports the hypothesis that the cysteine-rich C-terminal halves of Cry1 proteins are essential for crystallization [16].

Up till now, the C-terminal half of Cry1A has been known as deprived of any toxicity and is digested by midgut proteases right after solubilization. Herein, we report a cryptic endotoxic character of *B. thuringiensis* Cry1Ab insecticidal crystal protein. The individual expression of either the insecticidal or crystal domain in *B. thuringiensis* carried out lethal effects on its growth. In contrast with the N-terminal insecticidal

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domain, the C-terminal portion of Cry1Ab protoxin was highly toxic to *Escherchia coli* and *Agrobacterium tumefaciens*. In spite of this toxicity, the complete protoxin was harmless. The toxicity of the C-terminal portion was also reduced or neutralized when a C-terminal was fused to domain III of the insecticidal N-terminal domain.

2. Materials and methods

2.1. Bacterial strains and culture media

Escherchia coli XL-1 Blue (Stratagene, CA) cells were grown in LB medium supplemented with ampicillin at 100 mg/L at 37 °C [17]. *A. tumefaciens* PGV 2260 bacteria were directly transformed according with Holster et al. [18] and selected on YEB medium supplemented with rifampicin at 50 mg/L, carbenicillin at 50 mg/L, ampicillin at 100 mg/L, kanamycin 100 mg/L at 28 °C. Acrystalliferous *B. thuringiensis* subspecies *israelensis* cry$^-$ bacteria were transformed by electroporation [19], and positive cells grown on solid LB medium supplemented with erythromycin at 25 mg/L. The GYS sporulation medium [0.1% glucose, 0.2% yeast extract, 0.05% K_2HPO_4, 0.2% $(NH4)_2SO_4$, 0.002% $MgSO_4$, 0.005% $MnSO_4$, and 0.008% $CaCl_2$] was used for growth curve and viability determinations [20].

2.2. Recombinant plasmid constructs

All genetic constructs are represented in Fig. 1. The 3 kb DNA fragment containing the C-terminal portion of *cry1Ab* gene (*cry1Ab cter*) was isolated by PCR from pOS 4403 plasmid, donated by Dr. Donald Dean, University of Ohio [21], using the following primers: 5′ gaggatccaggcctgcagaagtaacctttgag 3′ (homologous to +1816 position into the *cry1Ab* gene) and 5′ ctgtgactggtgagtactcaaccaagg 3′ (inside of ampicillin resistant gene). Subsequently, the *Bam*HI–*Pst*I-digested amplicon (2064 pb) was inserted into pBlueScrip SK (+) plasmid to produce the pBS-*cry1Ab*-cter, and sequencing was determined by the dideoxynucleotide-based method. The pCons 36 and pMal-ter plasmids were generated by inserting the *Bam*HI–*Pst*I *cry1Ab-cter* gene segment into the pQE-30 (Qiagen, CA) and pMal-p2 (NE Biolabs, ME) expression vectors. Additionally, a 2.6 Kb *Sac*I–*Pst*I DNA segment containing the domain III of the insecticidal toxin together with its C-terminal portion, was isolated from pOS4403 and inserted into the pQE-30. The resulting plasmid was named pCons SP. Recombinant expression of the Cry1Ab C-terminal protein fused to maltose binding protein (MBP) in *A. tumefaciens* was carried out with the pGTox plasmid generated by insertion of a 3.6 kb *Fsp* I DNA fragment from pMal-ter into the *Sma*I site of binary vector pCAMBIA 1301 (http://www.cambia.org/). This DNA fragment contained both

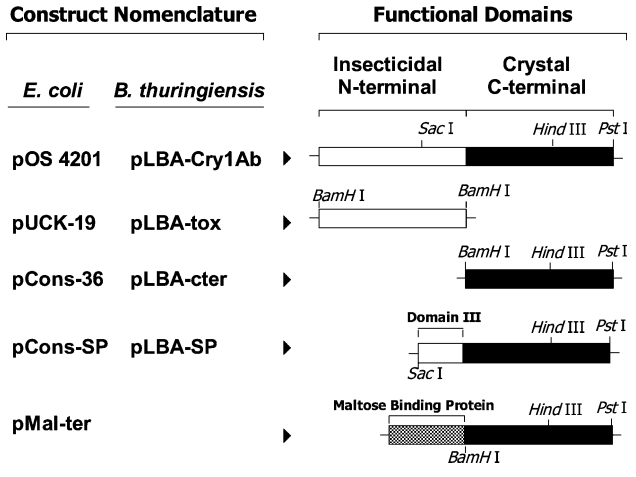

Fig. 1. Diagram of the genetic constructs used in this study. All genes expressed in *B. thuringiensis* were under the control of *cry1Ac* promoter in pLBA-100 plasmid. Genes expressed in *E. coli* were under the control of Ptac promoter except for pUCK-19, where the Plac promoter was used instead.

the *mbp-cry1Ab-cter* gene and the *lacIQ* repressor. For recombinant gene expression in *B. thuringiensis*, the binary plasmid pLBA-100 was constructed as follows: Briefly, the promoter and transcriptional terminator regions of *cry1Ac* gene were amplified from *B. thuringiensis* HD-73 total DNA using 5′-aaggtgaattccaggtaaatggttctaac-3′ (forward) and 5′-gatagaattcctccatctcttttattaag-3′ (reverse) as primers for the promoter, and 5′-ctcaagcttactcaggtttaaatatcg-3′ (forward) and 5′-ttcaagcttcaaaaacatcctattt-3′ (reverse) as primers for the terminator. The *cry1Ac* gene terminator and promoter regions were inserted, respectively, into the *Hin*dIII and *Eco*RI sites of pTH304 binary vector, donated by Dr. Didier Lereclus from Institut Pasteur, Paris [22]. Subsequently, the following plasmids were made using the pLBA-100: (1) pLBA-tCry1Ab by insertion of *Bam*HI fragment from pUCK19 [23]; (2) pLBA-Cry1Ab-cter by insertion of *Sma*I–*Bam*HI *cry1Ab-cter* fragment from pBS-*cry1Ab*-cter; (3) pLBA-Cry1Ab by insertion of *Bam*HI *cry1Ab* gene from pOS 4403; and (4) pLBA-SP by insertion of *Sac*I–*Pst*I fragment from pCons-SP. Finally, the *cry1ab-cter* gene was split in two by *Hin*dIII digestion of pCons36 plasmid to generate the pCons Δ*Hin*dIII and pCons *Hin*dIII.

2.3. Growth curves and viability assays

Individual colonies were grown separately overnight in 2 ml of the medium supplemented with antibiotics. Next day, fresh cultures were re-inoculated and grown to a cell density of 0.4 OD. At this point, IPTG was added to a final concentration of 1 mM for induction of recombinant protein expression in *E. coli* and *A. Tumefaciens*. The IPTG is not required for protein expression in *B. thuringiensis* cells. OD readings (OD 600 nm), viability and protein expression were determined at 0, 30, 60, 90, 120, 150, 180, and 210 min. Viability of cells was estimated by ability to form colony-forming units (CFU) on solid media. *B. thuringiensis* samples were boiled at 96 °C for 20 min before plating. The number of colonies emanating from spores or viable cells were counted after 24 h of incubation. Each point represents a mean of duplicate experiments using three different dilutions.

2.4. Purification of Cry1Ab-cter protein, antibody generation and Western blot analysis

Sonication was used for cell disruption and inclusion bodies were solubilized in buffer A (0.01 M Tris–HCl, 0.1 M Na_2PO_4, 6 M Urea, pH 8). Supernatants were then loaded onto a Chelating Sepharose column (Pharmacia Biotech, Sweden) and Cry1Ab C-terminal portion was eluted using buffer A containing Imidazol 250 mM. The eluted protein was dialyzed against 0.1 M Na_2CO_3/$NaHCO_3$/0.1% of glycerol, pH 9.6. Purity and integrity of the recombinant protein was analyzed on SDS–PAGE [24]. Protein concentration was determined by the Bradford method [25]. For immunization in rabbits, purified Cry1Ab-C-terminal was administered subcutaneously in complete or incomplete Freund's adjuvants [26]. Specific polyclonal antibodies were immunopurified using a Cry1Ab C-terminal Sepharose column. Immunoreactivity and specificity of polyclonal antisera were measured by ELISA in 96-well plates coated with 100 μl (10 μg/ml) of *B. thuringiensis* HD73 Cry1Ac toxin or recombinant Cry1Ab C-terminal in carbonate buffer, pH 9.6 [27]. This anti-serum did not cross-react with the N-terminal part of the protoxin. Proteins were electro-transferred from SDS–PAGE gels onto nitrocellulose membranes and exposed to the generated Cry1Ab C-terminal-specific antiserum. Immune complexes were detected using an ECL-Western blot kit (Amersham-Pharmacia).

2.5. Protein analysis

Recombinant bacterial cells harboring each of the expression plasmids were grown in specific media with antibiotics. At regular time intervals post-induction (0, 2, 4, 6, 10, 12 h), aliquots (30 μl) were taken and mixed with 30 μl of SDS–PAGE loading buffer, then boiled for 5 min, cooled and spun down at $12\,000 \times g$ for 10 min. The cleared supernatants were analyzed on SDS–PAGE-10% and total protein profiles revealed with Coomassie brilliant blue.

2.6. Solubility analysis

E. coli cells were harvested 2 h after IPTG-induction from a 200-ml culture, resuspended in 20 ml of TE 1× and disrupted by sonicating twice in a Branson Sonicator 250. Inclusion bodies were collected by centrifugation at $6000 \times g$ for 10 min. These inclusion bodies yielded about 4 mg of recombinant protein per liter of culture. Isolated inclusion bodies (30 μg protein) were resuspended in 36 μl of universal

buffer at increasing pH ranging from 5 to 12 with or without 1% of β-mercaptoethanol. After overnight incubation, suspensions were centrifuged, then supernatants and pellets were resolved by electrophoresis on a 10% SDS–PAGE.

2.7. Calculations and statistics

Each value represents the mean of three independent experiments. Maximal variation, expressed as a variation coefficient, is given in the legend where applicable.

3. Results

3.1. Cry1Ab C-terminal crystal domain expression in E. coli

The C-terminal part of *cry1Ab* gene [encoded between P606-E1156] was N-terminal fused to a 6-Histidine tag under the control of the synthetic, IPTG-sensitive Ptac promoter. The expected 65 kD recombinant protein was detectable as early as 60 min post-induction (Fig. 2A and B). This recombinant protein was unstable after 12 h becoming undetectable thereafter (Fig. 2B), most likely by intracellular proteolysis. Furthermore, the Cry1Ab C-terminal also formed inclusion bodies, which resembled the bipyramidal crystal produced by *B. thuringiensis* during sporulation (Fig. 2E). The identity of these recombinant peptides was confirmed by Western blot using a previously generated anti Cry1Ab C-terminal rabbit antiserum (data not shown).

Since the expression of Cry1Ab C-terminal in *E. coli* was fading with time, we checked toxicity of this protein to the host bacteria. Temporal expression of Cry1Ab C-terminal showed a lethal effect on *E. coli*. After transcriptional induction with IPTG, the growth of the cells bearing the pCons36 plasmid (encoding for Cry1Ab C-terminal) was totally inhibited getting the cultures into a forced stationary phase (2 h after induction), while the number of viable cells dropped dramatically (Fig. 2C and D). Fusing the Cry1Ab C-terminal to a non-specific peptide such as MBP did not reduce the toxicity. However, either insertion of the whole insecticidal domain or part of it (domain III encoded between R449 and P606) diminished such a lethal effect. No toxicity was found upon IPTG treatment in cells bearing empty vectors or expressing the insecticidal portion alone. Similar observations were made using solid media (Fig. 2F). It is noteworthy that purified Cry1Ab C-terminal added to liquid media did not have any inhibitory effect on *E. coli* growth (data not shown), which excludes any bystander effect.

3.2. Cry1Ab C-terminal crystal domain expression in A. tumefaciens

To confirm if Cry1Ab C-terminal portion has a similar toxic effect on other gram-negative bacteria, *mbp-cry1Ab-cter* gene together with *lacIq* were inserted into the pCAMBIA 1301

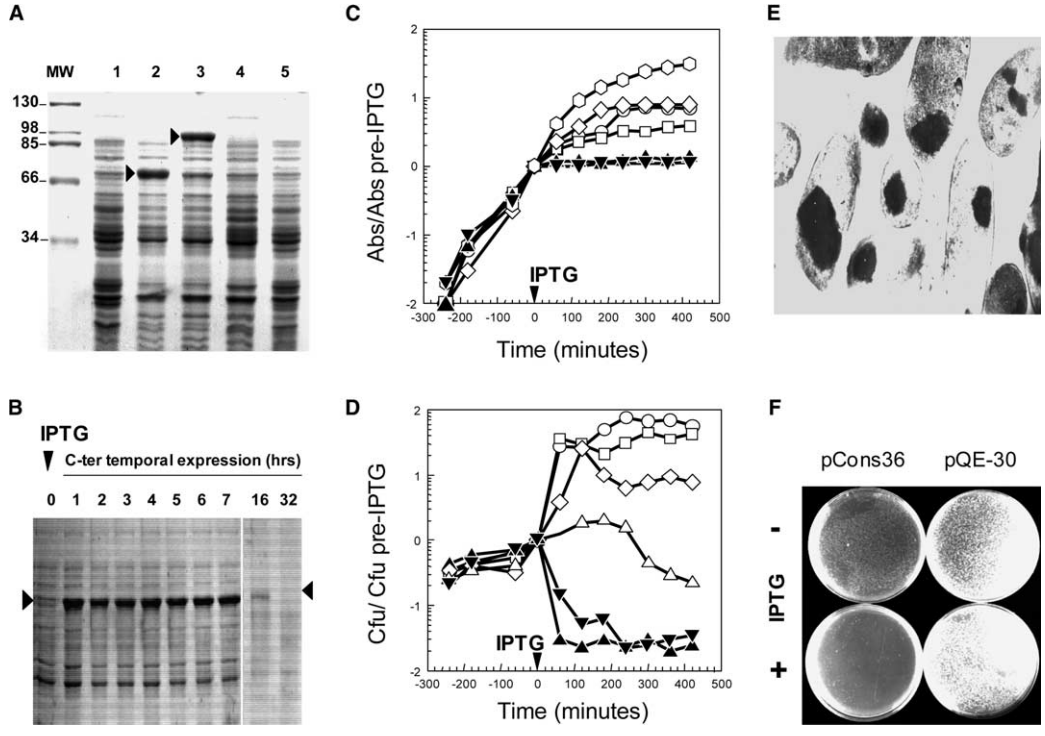

Fig. 2. Cry1Ab C-terminal crystal domain expression in *E. coli* cells. (A) Shown is the total protein profiles of IPTG-induced *E. coli* cells producing the following proteins (encoding plasmids between parentheses): (1) Empty vector (pQE-30); (2) Cry1Ab C-ter (pCon36); (3) MBP-Cry1Ab-Cter (pMal-ter); (4) tCry1Ab (pUCK-19); (5) DomainIII-C-terminal (pCon-SP). Total protein lysates were resolved on a 10% SDS–PAGE. Only recombinant proteins that were overexpressed were marked with arrows. (B) Temporal expression of Cry1Ab C-terminal in *E. coli showed by SDS–PAGE*. Note that this expression fades after 16 h of induction. Time 0 corresponds to the time when the inducter (IPTG) was added. (C) and (D) Respective growth curves and viability of *E. coli* transfected with one of the following plasmids: pQE 30 (empty vector, -◯-); pOS4201 (Cry1Ab, -□-); pCons36 (Cry1Ab-C-terminal, -▲-); pMal-ter (MBP-Cry1Ab-C-terminal, -▼-), pMAl-c (MBP, -◇-) and pConsSP (DIII-cter, -△-). The expression of Cry1Ab C-terminal alone or fused to MBP protein was lethal for *E. coli*. All values are expressed with respect to the absorbance (A_0) or amount of colonies (CFU_0) at the induction time (arrowhead). All standard errors of three individual experiments were less than 30%. (E) Electronic microphotograph of "ghost" *E. coli* cells containing the bipyramidal-like inclusion bodies formed by Cry1Ab C-terminal. (F) Representative experiment to show the lethal effect of Cry1Ab C-terminal expression (bottom left plate) in *E. coli* cells grown on LB agar-based medium. These media were supplemented with or without IPTG. The empty vector pQE-30 was used as negative control.

binary vector and introduced into *A. tumefaciens* by direct transformation. The recombinant protein was expressed efficiently in this bacterium after IPTG addition (Fig. 3C). The expression of Cry1Ab-pMal-cter inhibited *A. tumefaciens* growth and markedly affected cell viability (Fig. 3A and B) in both liquid and solid media (Fig. 3D), thus reproducing its effects on *E. coli*.

3.3. *Cry1Ab C-terminal crystal domain expression in* B. thuringiensis

To ascertain whether the production of Cry1Ab C-terminal is also toxic to its natural host *B. thuringiensis*, we cloned *cry1Ab-cter* gene, and a portion encoding the insecticidal domain only, under the control of the active Cry1Ac promoter during sporulation (constructs outlined in Fig. 1). Both vectors were able to inhibit *B. thuringiensis* growth in GYS sporulation media, which usually occurs within the first 10 h of culture. Moreover, those cells were not able to produce viable spores. In contrast, production of the whole protoxin containing both the insecticidal and the C-terminal domains, did not lead to any signs of toxicity (Fig. 4A and B). Toxicity was lost when the insecticidal domain III was fused with the Cry1Ab C-terminal. Also, no toxicity was seen when *B. thuringiensis* cells were transfected with the empty vector pLBA 100.

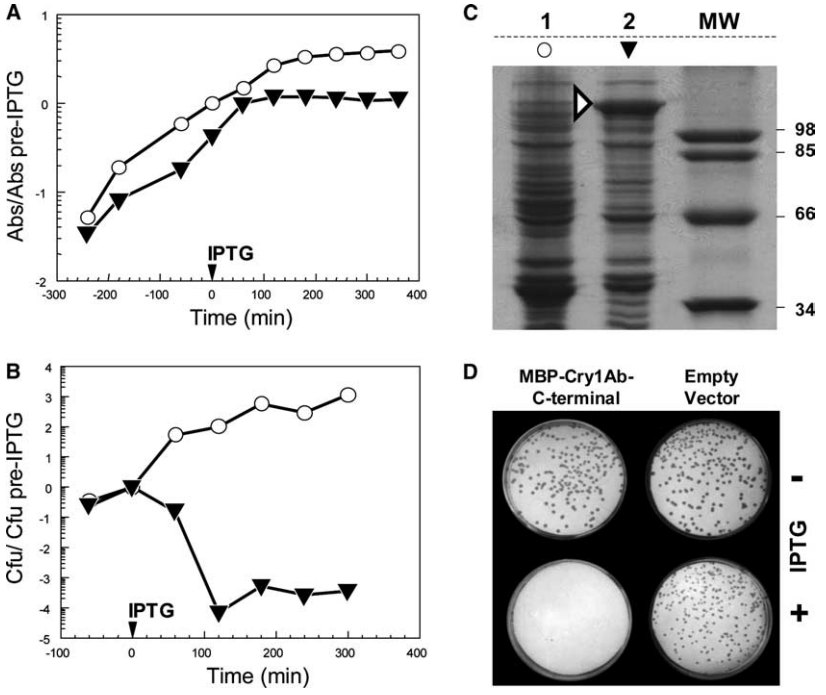

Fig. 3. Cry1Ab C-terminal expression in *A. tumefaciens* cells. (A) and (B) Respective growth curves and viability of *A. tumefaciens* cells expressing Cry1Ab MBP-C-terminal protein (pCAMBIA-C-ter, -▼-). The control was made of the empty vector pCAMBIA 1301 (-◯-). Note that this expression was also lethal to *A. tumefaciens* cells. IPTG-induction point was mark with an arrowhead. Standard errors of the three individual experiments were less than 25%. (C) SDS–PAGE analysis of Cry1Ab MBP-C-terminal production in *A. tumefaciens*. This protein was efficiently produced in those cells after induction with IPTG (arrowhead). (D) Representative experiment that shows the lethal effect of Cry1Ab MBP C-terminal in *A. tumefaciens* cells was grown on LB agar-based medium (bottom left plate). These media were supplemented with or without IPTG. The empty vector pCAMBIA 1301 was used as negative control.

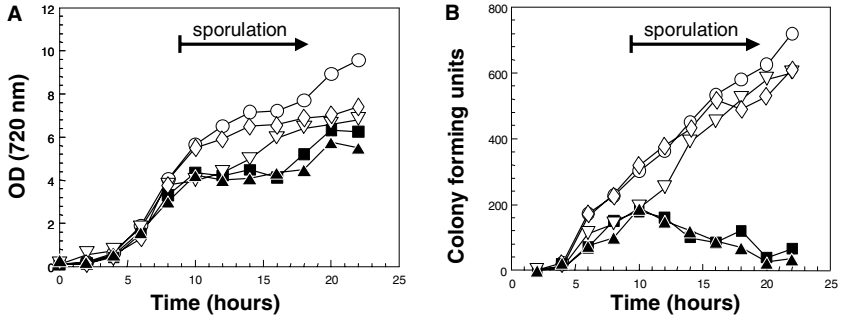

Fig. 4. Cry1Ab C-terminal expression in *B. thuringiensis* cells. (A) and (B) Respective growth and viability curves of *B. thuringiensis* producing the following recombinant protein: DIII-cter (pLBA SP, -◯-); Cry1Ab (pLBA Cry1Ab, -◇-); Cry1Ab C-terminal (pLBA-ter, -▲-), Cry1Ab-tox (pLBA-tox, -■-) and an empty vector (pTH304, -▽-). Note that both Cry1Ab C-terminal and Cry1Ab N-terminal were highly toxic to *B. thuringiensis* after sporulation, sporulation generally starts 8–12 h after seeding. Standard errors of the three individual experiments were less than 35%.

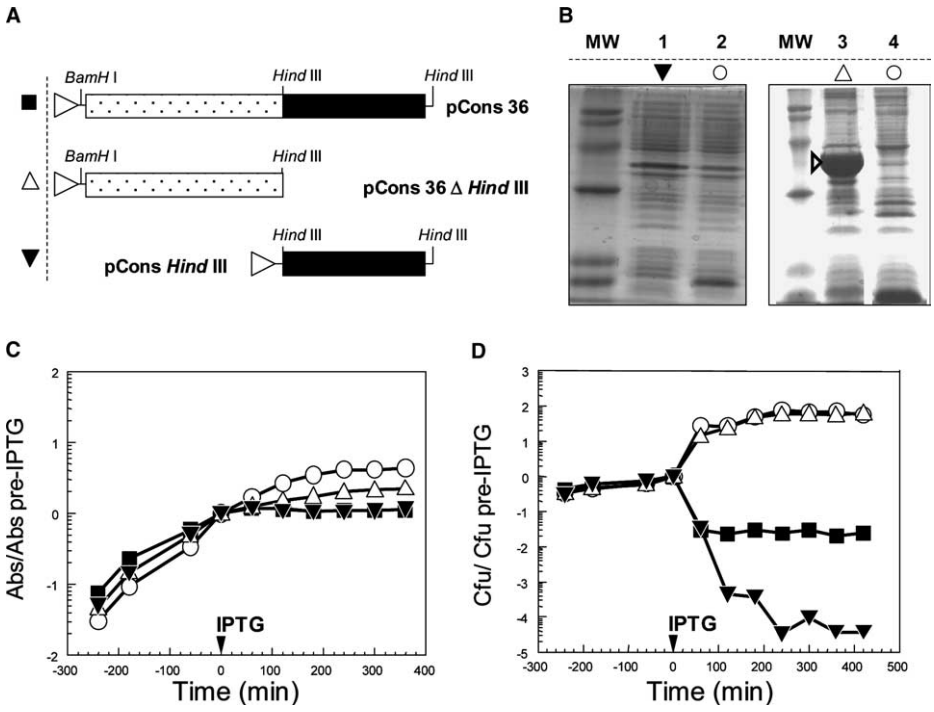

Fig. 5. (A) Genetic constructs were used to determine the toxic region of Cry1Ab C-terminal to *E. coli*. (B) SDS–PAGE analysis of the expression of pCons*Hin*dIII (lane 1) and pConsΔ*Hin*dIII (lane 3); the latter being the only portion that was efficiently produced. Empty vectors pQE-30 (lane 4) and pQE-32 (lane 2) were used as negative controls. (C) and (D) Respective growth and viability curves of *E. coli* cells producing the following recombinant peptides: with empty vector (pQE-30, -○-); Cry1Ab-cter (pCons36, -■-); Cry1Ab-Δ cter (pConsΔ*Hin*dIII, -△-) and Cry1Ab-cter-III (pCons*Hin*dIII, -▼-). Note that the expression of Cry1Ab C-terminal-*Hin*dIII was toxic to *E. coli* cells. The induction point was marked with an arrowhead. Standard errors of the three individual experiments were less than 15%.

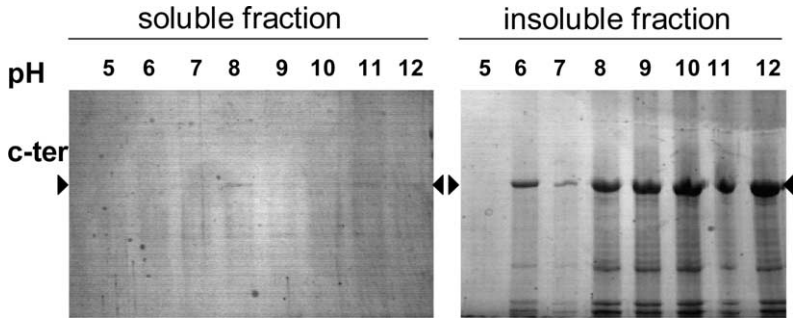

Fig. 6. pH–solubility of Cry1Ab inclusion bodies is not dependent on the C-terminal portion. Inclusion bodies were produced in *E. coli* were isolated by differential centrifugation and incubated in different pH buffers in presence or absence of β-mercaptoethanol (1% v/v). No C-terminal portion was recovered in the soluble fractions.

3.4. Localization of the toxic region within the Cry1Ab C-terminal domain

To locate the toxic region of the Cry1Ab C-terminal in *E. coli*, we split this gene in two segments that encode for the following two polypeptides: (i) a 37.2 kDa protein [from P606-R914, *cons*Δ *Hin*dIII], and (ii) the far C-terminal part of the protoxin [from E915-E1156, *cons Hin*dIII] (Fig. 5A). Only this first part of the protein was efficiently produced, and showed to be harmless to *E. coli* host cells. In contrast, the part encoded by Cons*Hin*dIII was produced in minute amounts and was extremely toxic to the host (Fig. 5C and D).

3.5. Solubility of Cry1Ab C-terminal inclusion bodies

Bipyramidal inclusion bodies formed by the whole Cry1Ab protoxin are known to have a peculiar solubility dependent on the environment's pH (solubilization generally occurring at pH > 9). Herein, we tested whether purified Cry1Ab C-terminal inclusion bodies in *E. coli* are able to keep these solubility properties. We found that Cry1Ab C-terminal inclusion bodies are resistant to solubilization even at pH > 9 (pH range 5–12) in presence (Fig. 6) or absence (data not shown) of β-mercaptoethanol as a reducing agent. Solubilization was only possible with high concentrations of Urea (6 M).

4. Discussion

The majority of Cry1A protoxins are composed of two functional segments [9]: the protease-resistant core toxin and the C-terminal portion whose function is not yet very well

understood. Studies in the past focused on determining the structure and mode of action of the insecticidal domain; only few have addressed other roles of Cry1A C-terminal domains besides crystal formation. Herein, we found that the N-terminal portion corresponding to the active insecticidal toxin fragment showed toxicity against the natural host *B. thuringiensis* but had none for the experimental *E. coli* recipient. More importantly, we produced the Cry1Ab C-terminal domain in three different bacterial hosts, and provided evidence that it had a cryptic toxic property towards not only the natural host *B. thuringiensis* but also to experimental recipients *E. coli* and *A. tumefaciens* as well. Hence, both parts of the protoxin sitting on the same protein are necessary to evade this effect. Based on these findings, we postulate that a natural dot/anti-dot relationship exists between the Cry1Ab two halves. The putative requirement for the dot/anti-dot system within the host may explain its high conservation among Cry subfamilies.

This is the first report that delineates another function for the Cry1Ab C-terminal portion other than crystal formation. From our findings, an important question arises on why other Cry proteins lack the C-terminal domain from their protoxin yet the latter are not toxic to their respective natural hosts. Indeed this fragment is not present in Cry3A, Cry3B, Cry3Bb, Cry3Ca, Cry2Aa, Cry2Ab, Cry2Ac, and Cry11Aa or is very small in some other protoxins such as Cry1IA and Cry1Ib (75 residues) and Cry13A (111 residues) [10]. The answer may lie in the extra-long N-terminal portions of the C-terminal-lacking protoxins, which may confer both crystal forming and antitoxic (anti-dot) properties. Nonetheless, firm conclusions may only be reached by generating mutants lacking those domains.

Besides having a toxic effect to its natural host *B. thuringiensis*, an equally important finding from this study is that production of Cry1Ab C-terminal also showed toxicity for two other gram-negative bacteria, namely *E. coli* and *A. tumefaciens*. Interestingly, Cry1Ab C terminal was produced promptly in *E. coli* and lasted for 12 h even though cell viability dropped quickly (100 min after induction). Moreover, electronic microscopy showed inclusion bodies inside ghost-like *E. coli* cells, much like the ones observed during bacterial ghost production by intracellular endonuclease expression [28]. The hypothesis that Cry1Ab C-terminal expression initially inactivates bacterial cell division but keeps the protein production and turnover processes is in agreement with our findings.

Structurally, the Cry1 protoxins show a striking asymmetry between the two halves in terms of cysteine composition and physical properties. This raises the question as to whether these two regions are functionally independent. Differential scanning calorimetry studies demonstrated that part of the insecticidal moiety of the protoxin must undergo conformational changes on activation, to adopt a more thermostable structure [7]. As we reported, the fact that domain III was enough to reduce the C-terminal-mediated toxicity, may lead to think that this part by itself could confer those conformational changes, making the protoxin safer for the host bacterium undergoing sporulation.

The Cry1A C-terminal domain by itself was able to form inclusion bodies featuring the ones formed by the protoxin in *E. coli*, whereas from our previous reports, we demonstrated that the N-terminal domain alone produced a soluble protein [23]. However, the solubility of the C-terminal was quite different from that of the whole protoxin. The C-terminal bodies were unable to dissolve even at high pH values in presence or absence of reducing agents. Those findings indicate that the crystal formation and the peculiar solubility features of the Cry1As are conferred by the structure of the protoxin where both domains take crucial roles. The generation of a chimeric protein by fusion of Cry3A (C-terminal lacking protoxin) to the Cry1Ab C-terminal portion gave rise to insoluble inclusion bodies in *E. coli* (data not shown). Thus, we propose that Cry1As need their terminal portion to conserve the solubility at high pH, as commonly found in lepidopteran midgut. This study also supports the idea that both protoxin moieties are required for proper folding needed for sequential protoxin proteolysis leading to the N-terminal half to acquire its insecticidal function [7]. Interestingly, decades ago Holmes and Monro [29], based on X-ray power diffraction of *B. thuringiensis* crystals concluded that the protoxin was an elongated molecule unlike the tertiary structure described for crystallized N-terminal domain.

Finally, Cry1Ab protoxin contains a remarkably high number of cysteine residues, 14 out of 16 being in the C-terminal half, which makes the observed rapid refolding of this region unusual. In the absence of reducing agents, cysteine-rich proteins are known to commonly form incorrect disulfide bridges [30]. A plausible explanation to this may be that thiol groups located on the surfaces of the protoxin are able to form symmetrical interchain disulphide bridges during crystal formation.

In conclusion, toxicity associated with individual production of the Cry1Ab halves (insecticidal N-terminal or C-terminal) uncovers a cryptic endotoxicity to the natural host *B. thuringiensis*. Acquisition of this property also reveals a fail-safe mechanism operating, protecting the host bacteria in a manner much like a dot/anti-dot system. Furthermore, our findings strongly support the assumption that *B. thuringiensis* Cry1A protoxin insecticidal and crystal forming domains do not operate independently, but rather have complementary roles for proper crystal formation, solubility properties, and shielding from endotoxicity.

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