Mol Biotechnol (2008) 38:121–127
DOI 10.1007/s12033-007-9015-6

RESEARCH

# Evidence of the Importance of the $Met^{115}$ for *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa Protein Cytolytic Activity in *Escherichia coli*

**Raida Zribi Zghal · Hana Trigui · Mamdouh Ben Ali · Samir Jaoua**

Published online: 8 November 2007


**Abstract** Cyt1Aa is a cytolytic toxin, found together with the delta-endotoxins in *Bacillus thuringiensis* subsp. *israelensis* parasporal insecticidal crystals. The latter are used as an environmental friendly insecticide against mosquitoes and black flies. Contrary to Cry delta-endotoxin, the mode of action of Cyt1Aa is not completely understood. In the absence of direct structural data, a novel mutated *cyt1Aa* gene was used to obtain indirect informations on Cyt1Aa conformation changes in the lipid membrane environment. A mutated *cyt1Aa* gene named *cyt1A97* has been isolated from a *B. thuringiensis israelensis* strain named BUPM97. The nucleotide sequence predicted a protein of 249 amino acids residues with a calculated molecular mass of 27 kDa. Both nucleotide and amino acid sequences similarity analysis revealed that *cyt1A97* presents one amino acid different from the native *cyt1Aa* gene. This mutation was located in the helix α C corresponding to a substitution of $Met^{115}$ by a Thr. The heterologous expression of the *cyt1A97* and another *cyt1Aa*-type gene called *cyt1A98*, not affected by such mutation used as control, was performed in *Escherichia coli*. It revealed that the mutated Cyt1A97 protein was over produced as inclusion bodies showing a very weak toxicity to *E. coli* contrarily to Cyt1A98 that stopped *E. coli* growth. Hence, hydrophobic residue Met at position 115 of Cyt1Aa should play a very important role for the maintenance of the structure and cytolytic functions of Cyt1Aa.

R. Z. Zghal · H. Trigui · S. Jaoua (✉)
Laboratory of Biopesticides, Centre of Biotechnology of Sfax, P.O. Box'K', 3038 Sfax, Tunisia
e-mail: Samir.Jaoua@cbs.rnrt.tn

M. B. Ali
Laboratory of Enzymes and Metabolites of Prokaryotes, Centre of Biotechnology of Sfax, P.O. Box'K', 3038 Sfax, Tunisia

**Keywords** *Bacillus thuringiensis* subsp. *israelensis* · *cyt1Aa* · Mutation · Heterologous expression · Sequencing

**Abbreviations**

| | |
|---|---|
| *B* | *Bacillus* |
| *E* | *Escherichia* |
| PCR | Polymerase Chain Reaction |
| Cyt | Cytolytic toxin |
| LB | Luria-Bertani medium |

## Introduction

Cytolytic (Cyt) endotoxins are produced by a minor group of *B. thuringiensis*, mostly in subsp. that are toxic to dipteran insects [1–3]. Cyt toxins exhibit a broad spectrum of activity against a variety of eukaryotic cells in vitro but are highly specific towards dipteran insects in vivo [4–6]. *B. thuringiensis* subsp. *israelensis* is the mosquitocidal subspecies most thoroughly studied. One of the major components of its toxin is the cytolytic endotoxin Cyt1Aa. The Cyt1Aa toxin is of particular interest because of its affinity for unsaturated fatty acids in the lipid layer of the membrane, whereas Cry and *B. sphaericus* Bin proteins target specific receptor molecules. The level of mosquito larvicidal activity of Cyt1Aa by itself is low [7–8]. Despite its relatively low mosquitocidal activity, Cyt1Aa was demonstrated to increase Cry protein toxicity in *B. thuringiensis* subsp. *israelensis* through synergism [7, 9, 10]. Cyt1A synergizes or suppresses resistance to Cry11Aa toxin by functioning as a membrane-bound receptor. Recent evidences suggested that Cyt synergizes or overcomes resistance to mosquitocidal-Cry proteins by functioning as a Cry membrane-bound receptor. These data

represent an example of an insect pathogenic bacterium that carries a toxin and also its functional receptor, promoting toxin binding to target membranes and toxicity. [11–14]. Moreover, expression of cloned Cyt1Aa is lethal to *Escherichia. coli* [15] and to *B. thuringiensis* subsp. *kurstaki* (CryB) cells [8). Loss of colony-forming ability without substantial lysis, associated with immediate inhibition of DNA synthesis, was observed after induction of recombinant *E. coli* cells [15]. The chromosome replication complex has been implicated as the target for Cyt1Aa in *E. coli* [15] because the membrane fraction attached to DNA is enriched in phosphatidylethanolamine [16], which is among those phospholipids known to preferentially interact with Cyt1Aa [17]. Thus, in *B. thuringiensis* subsp. *israelensis* both Orf2 and P20 may act as chaperones to initiate, facilitate or stabilize crystal formation in their original hosts, and protect *E. coli* and *B. thuringiensis* strains from lethal action of Cyt1Aa. The detailed mechanism in which the Cyt toxins damage the target cell is still under debate. It could act as a pore former as described previously [18] or kill the cell via a detergent-like mechanism as suggested by Manceva et al. [19]. Prediction of the Cyt1Aa structure became possible only when Li et al. [20] determined the X-ray structure of the related *B. thuringiensis* toxin Cyt2Aa1 (CytB) based on sequence analysis, hydrophobicity and mutagenesis data. The three-dimensional structure of CytB was determined [20], and found to have a single domain of $\alpha/\beta$ architecture [20]. Similar to Cyt2Aa1, Cyt1Aa1 appears to consist of two outer $\alpha$-helix hairpins (helices A-B and C-D) flanking a core of mixed $\beta$-sheet (strands 1–7) [21]. Based on this structure, plus the previous work on biochemical characterization of the toxin mutant and the results of proteolysis, Li et al. [20] proposed that it is the $\beta$-sheets rather than the $\alpha$-helix that are responsible for membrane binding and pore formation. Whereas, Gazit and Shai [22] demonstrated that helix-1 and helix-2 are involved in the toxic mechanism of Cyt1Aa toxin of *B. thuringiensis* subsp. *israelensis*. Both these hydrophobic helical segments may facilitate pore formation by the toxin, with a predominant contribution by helix-2 [22]. In fact, that helix-2 can permeate phopholipid vesicules better than helix-1, may be due to its higher ability to form aggregates within the membrane, as revealed from their binding isotherm. Characterization of the toxin structure in the membrane-bound state, is however the key to understanding its biological function in a membrane. A conformational change of the toxin upon its binding to the membrane was observed [20]. Current evidence suggests that the helical hairpin, containing $\alpha$ C, is the most likely machinery for membrane insertion and channel formation [20]. Thus, in the present work, we evidenced the importance of the $Met^{115}$ of Cyt1Aa helix $\alpha$ C of *B. thuringiensis israelensis* for its cytolytic activity in *E. coli*.

## Materials and Methods

### Bacterial Strains, Plasmids and Growth Conditions

*Bacillus. thuringiensis* subsp. *israelensis* BUPM97 was isolated from a Tunisian soil in the laboratory according to Jaoua et al. [23]. This strain was selected for its insecticidal activity [24] against larvae of several dipterans insects such as *Culex pipiens*. *B. thuringiensis* subsp. *israelensis* BUPM98 was used as reference strain. For routine use in the laboratory, isolates were grown in Luria-Bertani (LB) medium at 30 °C with shaking at 200 rev $min^{-1}$, and for long storage, the isolates were stored as glycerol stocks at (80 °C). *E. coli* strain named pMOS*Blue* cells (*end*A1 hsdR17 ($r_{k12}-m^{+}_{k12}$) *supE44thi*-1 *rec*A1 gyrA96 *rel*A1 lac[F′ *pro*$A^{+}B^{+}$ lac$I^{q}$ZΔM15: Tn10($Tc^{R}$)]) was obtained from Amersham (Paris, France) and used as the host organism for recombinant DNA cloning and heterologous expression. Plasmid pMOS*Blue* (Amersham, Paris, France) and pBADGFPuv (Clontech; GenBank accession no. U62637) were used in this study as cloning vectors and expression vector. pBADGFPuv plasmid is a vector that was used for substituting *gfp* gene by other genes for expressing downstream arabinose operon promoter ($P_{BAD}$) [25]. pBAD*cyt1A98* is a recombinant vector expressing a normal *B. thuringiensis israelensis cyt1Aa* gene downstream $P_{BAD}$ and used control for Cyt1A97 cytolytic activity assessment.

### DNA Extraction and PCR Amplification

Plasmid DNA was extracted from *B. thuringiensis* strains using the alkali lysis method including a lysosyme treatment step [26]. The oligonucleotides used in this study were synthesized by the "Centre de Génétique Moléculaire, CNRS, GENSET, Orsay, France". Primers used to detect and to clone *cyt*-like gene were designed based on the published sequence *cyt1A(a)* gene [GenBank accession no. AL731825]. DNA extracted from *B. thuringiensis* strains was used as template for the PCR using a "Gene Amp PCR System 2700" (Applied Biosystems). PCR was accomplished as described by Jaoua et al. [23].

### Cloning and Sequencing of *cyt1A97* from BUPM97 and Transformation Procedures

The gene *cyt1Aa*, designed *cyt1A97*, was amplified by PCR using total DNA isolated from strain BUPM97 as template, Taq DNA polymerase (Amersham, Paris France) and primers D23 (5′GTTGTAAGCTTATGGAAAAT3′) and D24 (5′TTAGAAGCTTCCATTAATA3′) complementary

to the regions located at the initiation codon (ATG) and the termination codon (TAA), respectively. The amplified fragment (0.75 kb) corresponding to the Open Reading Frame (ORF) of the *cyt*-gene was purified from the agarose gel with MiniElute Gel Extraction Kit (Qiagen S.A. France) and cloned in pMOS*Blue* vector generating a recombinant plasmid pMOS*cyt1A97*. Ligation and transformation of pMOS*Blue* cells was performed according to the manufacturer's protocol (Amersham, Paris France). Selection of *E. coli* pMOS*Blue* cells transformants was performed on LB medium plates containing ampicillin (100 μg $ml^{-1}$). The sequencing of the *cyt1A97* ORF was carried out using a taq DyeDeoxy Terminator Cycle Sequencing kit and a 3,700 ABI Prism DNA sequencer (Applied Biosystems, Foster City, Calif., USA) according to the instructions of the manufacturer. The cloned fragment sequence of approximately 750 bp was determined using the universal primer. To be certain of the real sequence, three PCR clones were used and the same sequence was obtained.

### Sequence Alignments

Sequence comparison with the data bases was performed using BLAST through the NCBI e-mail server. Multiple alignments were carried out using CLUSTALW programme [27] to generate a multiple sequence alignment of the selected protein sequences. The sequence of *cyt1A97* has been deposited in the GeneBank data base under the accession No. EF656359.

### Heterologous Expression of *cyt1A97* in *E. coli*

Over-expression of *cyt1A97* was achieved by cloning its ORF in the pBADGFPuv vector [25]. In this vector, expression is under control of the arabinose operon promoter ($P_{BAD}$), which can be induced in the presence of L-arabinose [25]. The NheI-EcoRI restriction fragment of this plasmid, containing the green fluorescent protein (GFP) coding sequence (positions 1,340–2,063), was replaced by an XbaI-EcoRI restriction fragment containing *cyt1A97* coding sequence obtained from the plasmid pMOS*cyt1A97*. This cloning step positioned the *cyt1A97* ORF just downstream of the $P_{BAD}$ promoter and allowed the expression of the latter in pMOS*Blue* cells. The recombinant plasmid named pBAD*cyt1A97* (6 kb) was transferred by transformation to pMOS*Blue* cells. Recombinant *E. coli* clones carrying the *cyt1A97* gene were grown at 37 °C overnight in LB broth supplemented with 100 μg of ampicillin per ml. This preculture was used to inoculate 50 ml of LB medium with an initial absorbance at 660 nm of 0.045. When the optical density reached 0.25 at 660 nm $P_{BAD}$ promoter was induced by L-arabinose (0.125 g $l^{-1}$ final concentration) and host cells were grown for 3 h post-induction at 37 °C and 200 rev $min^{-1}$. Then, cells were harvested by centrifugation at 2,400 × g for 10 min. The pellet was washed and suspended in distilled water and then analysed by sodium dodecyl sulphate (10% w/v) polyacrylamide gel electrophoresis (SDS-PAGE).

### Protein Modelling

Structure model of Cyt1A97 as well as Cyt1A98 were generated using the structure of the mosquitocidal delta-endotoxin CytB from *Bacillus thuringiensis* subsp. *kyushuensis* as template [20]. (pdb-ID:1cby) was determined by X-ray crystallography and solved at 2.6Å. The generated models were performed by the automated protein structure homology-modelling server SWISS-MODEL [28]. Finally, PyMOL [29] was used to render figures.

Concerning the Side chain modelling, the reconstruction of the model side chains is based on the weighted positions of corresponding residues in the template structures. Starting with conserved residues, the model side chains are built by iso-sterically replacing template structure side chains. Possible side chain conformations are selected from a backbone dependent rotamer library, which has been constructed carefully taking into account the quality of the source structures. A scoring function assessing favourable interactions (hydrogen bonds, disulphide bridges) and unfavourably close contacts is applied to select the most likely conformation. So there are no problems with side chain locations and interactions in generated models, and so model built structure will generally provide a useful basis for structure analysis.

## Results

### Cloning of the *cyt1Aa*-type Gene from the *B. thuringiensis* subsp. *israelensis* Strain BUPM97

Due to the interest provided by the *cry* genes rearrangement we had evidenced in *B. thuringiensis* subsp. *israelensis* strain BUPM97 [24], the investigation of the other components of the crystal, Cyt1Aa, was carried out. A PCR fragment of 750 bp containing the ORF of *cyt1A97* was cloned in the vector pMOS*Blue*. The resultant recombinant plasmid designated pMOS*cyt1A97* was used for the sequencing of the *cyt1A97* gene. The obtained sequence corresponds to an ORF of 750 bp encoding a Cyt1Aa protein of 249 amino acid residues. The predicted molecular mass of this protein was 27 kDa. The search for sequence

similarity of *cyt1A97* with the up to now known genes, using the Blast programme and multiple alignments using CLUSTALW programme (Fig. 1), showed that when compared with all known *cyt1Aa*-type genes, there was a substitution at position 342 of T to A (transversion) without any change in the amino acid sequence and another substitution at position 344 of T to C (transition) resulting in the substitution of $Met^{115}$ for Thr located in helix-2 of Cyt1Aa.

## Heterologous Expression of *cyt1A97* in *E. coli*

To study its expression, *cyt1A97* was cloned in the pBAD vector. The gene *cyt1A97* was cloned downstream of the strong $P_{BAD}$ promoter in the pBADGFPuv vector. Microscopic examination of recombinant transformants *E. coli* pMOS*Blue* cells (pBAD*cyt1A97*) revealed the presence of small inclusions (data not shown). The pellet of the recombinant cells was analysed by SDS-polyacrylamide gel electrophoresis (Fig. 2), using as control *cyt1Aa*-gene of the reference strain *B. thuringiensis israelensis* BUPM98, cloned and expected in pMOS*Blue* cells, in the same vector (data not shown). The SDS-PAGE analysis showed the presence of the expected 27 kDa protein (Fig. 2) corresponding to Cyt1A97 protein. These results demonstrate that Cyt1A97 originating from *B. thuringiensis* subsp. *israelensis* was well expressed in *E. coli*, contrary to Cyt1A98 which was not detected in the SDS-PAGE.

## Effect of a Single Mutation in Cyt1A97 on its Cytotoxicity

To further investigate the effect of the substitution $Met^{115}$ Thr of Cyt1A97, the growth kinetics of pMOS*Blue* cells

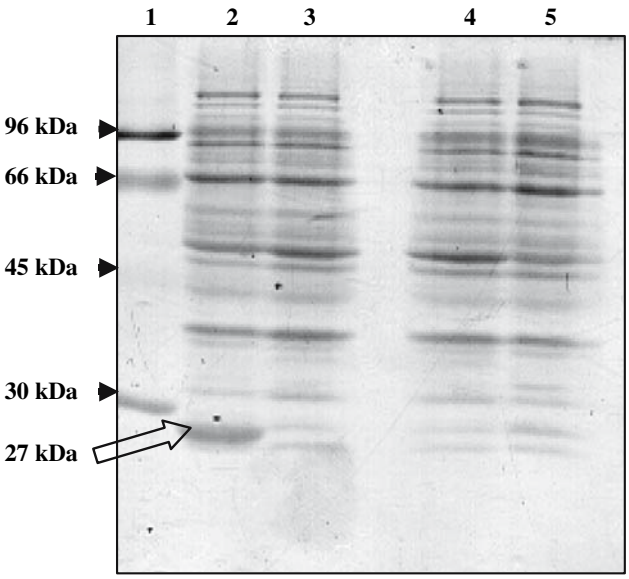


**Fig. 2** Heterologous expression of Cyt1A97 in *E. coli*: SDS–PAGE analysis. Lanes: 1, molecular weight markers (RPN 800, Amersham, Paris France): 96, 66, 45, 30, 20.1 and 14 kDa; 2, pMOS*Blue* cells (pBAD*cyt1A97*) induced with arabinose; 3, pMOS*Blue* cells (pBAD-*cyt1A97*) uninduced; 4, pMOS*Blue* cells (pBAD*cyt1A98*) uninduced; 5, pMOS*Blue* cells (pBAD*cyt1A98*) induced with arabinose

(pBAD*cyt1A97*), and that of pMOS*Blue* cells (pBAD-*cyt1A98*) used as positive control of cytolytic activity, were performed. The growth kinetics of the strain expressing Cyt1A97 was compared to that expressing the Cyt1A98 that is not affected by the mutation $Met^{115}$ Thr (Fig. 3). The mutant *cyt1A97* gene was highly expressed as inclusion bodies in *E. coli* cells. However, the recombinant *E. coli* cells expressing the native non-mutated *cyt1A98* gene delayed cell division just after induction. These results reveal that the substitution $Met^{115}$ Thr affected the cytotoxicity potential of Cyt1A97 in *E. coli* and demonstrate

**Fig. 1** Alignment of the amino acids sequences of Cyt1A97, *B. thuringiensis israelensis* Cyt1Aa and *B. thuringiensis kyushuensis* CytB, proteins using CLUSTALW programme. In helix-α-C, mutation $Met^{115}$ Thr was marked with an arrow

```
Cyt1A97         --MENLNHCPLEDIKVNPWKTPQSTARVITLRVEDPNEINNLLSINEIDNPNYILQAIML 58
cyt1Aa          --MENLNHCPLEDIKVNPWKTPQSTARVITLRVEDPNEINNLLSINEIDNPNYILQAIML 58
CytB            MYTKNFSNSRMEVKGNNGCSAPIIRKPFKHIVLTVPSSDLDNFNTVFYVQPQYINQALHL 60
                   :*:.:. :*    *  .:*     .  : :  *..  : :.     :*:** **: *

Cyt1A97         ANAFQNALVPTSTDFGDALRFSMAKGLEIANTITPMGAVVSYVDQNVTQTNNQVSVTINK 118
cyt1Aa          ANAFQNALVPTSTDFGDALRFSMPKGLEIANTITPMGAVVSYVDQNVTQTNNQVSVMINK 118
CytB            ANAFQGAIDPLN------LNFNFEKALQIANGIPNS-AIVKTLNQSVIQQTVEISVMVEQ 113
                *****.*: * .      *.*.: *.*:*** *.   *:*. ::*.* * . ::** :::

Cyt1A97         VLEVLKTVLGVALSG-SVIDQLTAAVTNTFTNLNTQKNEAWIFWGKETANQTNYTYNVLF 177
cyt1Aa          VLEVLKTVLGVALSG-SVIDQLTAAVTNTFTNLNTQKNEAWIFWGKETANQTNYTYNVLF 177
CytB            LKKIIQEVLGLVINSTSFWNSVEATIKGTFTNLDTQIDEAWIFWHSLSAHNTSYYYNILF 173
                : :::: ***:.:.. *. :.: *:::.*****:** :****** . :*::*.* **:**

Cyt1A97         AIQNAQTGGVMYCVPVGFEIKVSAVKEQVLFFTIQDSASYNVNIQSLKFAQPLVSSSQYP 237
cyt1Aa          AIQNAQTGGVMYCVPVGFEIKVSAVKEQVLFFTIQDSASYNVNIQSLKFAQPLVSSSQYP 237
CytB            SIQNEDTGAVMAVLPLAFEVSVDVEKQKVLFFTIKDSARYEVKMKALTLVQALHSSN-AP 232
                :*** :**.**  :*:.**:.*.. *::******:*** *:*::::*.:.*.* **.  *

Cyt1A97         IADLTSAINGTL--------------- 249
cyt1Aa          IADLTSAINGTL--------------- 249
CytB            IVDIFNVNNYNLYHSNHKIIQNLNLSN 259
                *.*: .. * .*
```

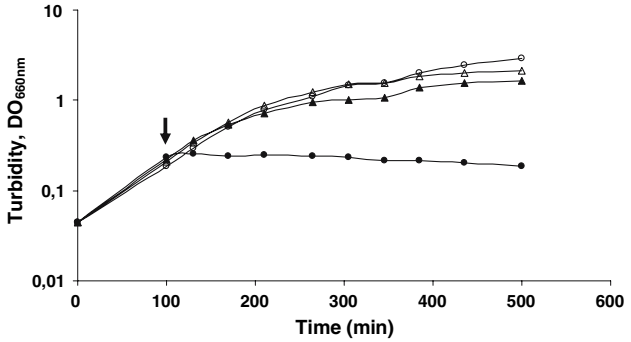


**Fig. 3** Growth kinetics curve in shake flask culture of *E. coli* pMOS*Blue* cells containing, respectively, *cyt1A97* and *cyt1A98*. △ pMOS*Blue* cells (pBAD*cyt1A97*) without induction; ▲ pMOS*Blue* cells (pBAD*cyt1A97*) induced with arabinose; ○ pMOS*Blue* cells (pBAD*cyt1A98*) without induction; ● pMOS*Blue* cells (pBAD-*cyt1A98*) induced with arabinose

well that $Met^{115}$ is essential for structural folding and cytolytic activity of Cyt1Aa.

### Effect of the $Met^{115}$ Thr Mutation on the Structure of Cyt1A97

To investigate the importance of the $Met^{115}$ Thr substitution at a molecular level, a three-dimensional model of Cyt1A97, was constructed on the basis of the crystal structure of CytB of *B. thuringiensis* subsp. *kyushuensis* [20] (Fig. 4a). The analysis of the generated model showed that Cyt1A97 and CytB share together the same structure as expected from their sequences identity (39%) [30, 31].

In fact, Cyt1A97 is a single domain protein of $\alpha/\beta$ architecture, with two outer layers of α-helices and a $\beta$-sheet in between. However, unlike most proteins of $\alpha/\beta$ architecture which contain $\beta\alpha\beta$ units, CytB shows an uncommon topology in that its helical layers consist of hairpins, which can be moved relative to the $\beta$-sheet without unravelling the sheet [20]. The helices αA, αB, αC and αD have an amphiphilic character, with hydrophobic residues packed against the $\beta$-sheet, and polar and charged residues on the molecular surface [20, 32]. Figure 4 illustrates the overall structure of Cyt1A97 and the position of $Thr^{115}$ in the top of the αC helix. In fact, the comparison between Cyt1A97 and Cyt1A98 focussed on $Met^{115}$ Thr region showed that $Thr^{115}$, establishes, by its hydroxyl group, an interaction with the carbonyl group of $Gln^{111}$ located in the boundary region between the sheet and the helix hairpin C-D (Fig. 4b). This interaction takes more importance when we know that conformational change in this region is important in the cytotoxic activity of both Cyt1A and CytB proteins [20]. Hence, any hindrance that affects this part of the protein will negatively affect its activity.

## Discussion

In the present work, we characterized another *cyt1Aa*-type gene cloned from a newly isolated strain BUPM97 of *B. thuringiensis israelensis* [33]. Contrary to the published *cyt1Aa*-type gene of *B. thuringiensis israelensis* strains, the coding sequence of *cyt1A97* is different by a substitution of $Met^{115}$ by Thr (Fig. 1). Regarding the effect of the reported substitution in *cyt1A97*, modifications in toxicity and cytolytic activity of the parasporal crystal of BUPM97 were expected.

The predicted model of Cyt1A97 structure, using the server SWISS-MODEL which is based on the X-ray structure of Cyt2Aa1 [20], revealed no modification in the three-dimensional structure. The question thus arises of its possible mode of action in bacteria. The leading hypothesis of Cyt1Aa's mode of action is that the toxin assembles into cation-selective channels in the membrane, through which ions equilibrate across the membrane, resulting in colloid-osmotic lysis and cell death. The protein is thought to partially insert into the membrane. Initial contact of Cyt1Aa with membranes involves electrostatic interactions of the charged amino acids, situated on its surface, with the lipid head groups. Electrostatic interactions between the charged amino acid residues and the lipid head groups are responsible for binding the protein to the membrane surface, while hydrophobic and/or van der Waals interactions make the membrane binding irreversible [34]. In helix-α C, only the role of three amino acids have been studied. $K^{118}$ and $E^{121}$ did not affect toxicity and lipid binding. $K^{124}$ affects toxicity and lipid binding [22, 33]. Structural investigation of Cyt1A97 shows that $Thr^{115}$ is located on the top of αC helix, pointing its side chain into the hydrophobic environment, made by the packaging of the hydrophobic residues of the helices against the $\beta$-sheet [20, 32]. As described by [20], results of the mutagenesis studies of Cyt1Aa imply a need for a conformational change in the membrane-bound toxin involving a hinge motion along the topological boundary between the $\beta$-sheet and the helix pair C-D. They proposed that the helices C-D are lifted off the sheet to lie on the surface of the membrane. This would expose the underlying hydrophobic face of the sheet and cause some part of the sheet to partition into the membrane core. The boundary region between the sheet and helix hairpin C-D is proposed to be the hinge for the conformational change that leaves the helices on the membrane surface while allowing part of the sheet to penetrate the hydrophobic zone of the membrane.

Comparison between Cyt1A97 and Cyt1A98 focussed on $Met^{115}$ Thr region showed that contrarily to $Met^{115}$, $Thr^{115}$ would establish by its hydroxyl group an interaction with the carbonyl group of $Gln^{111}$ located in the boundary region between the sheet and the helix hairpin

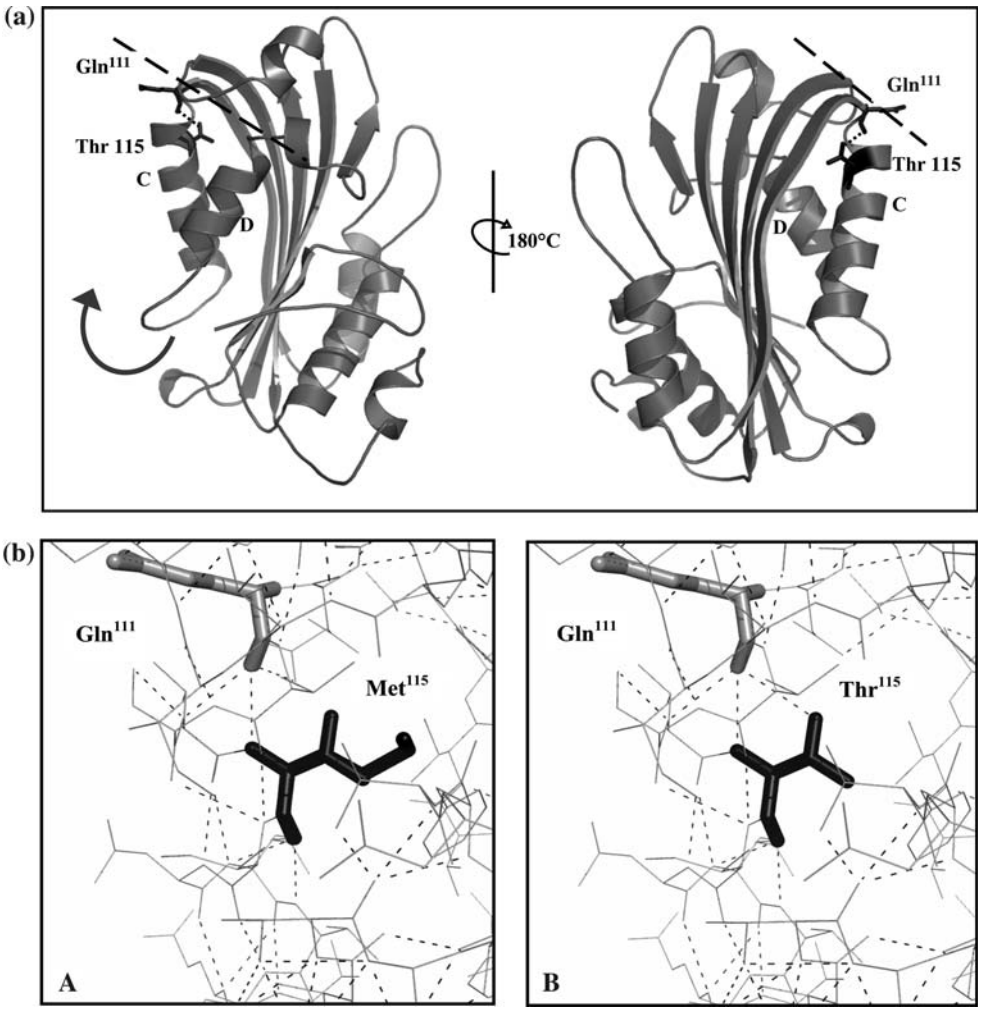


**Fig. 4** (**a**) Schematic ribbon diagram of Cyt1A97 structure, (dashed lines): The boundary region between the sheet and helix hairpin C-D, proposed to be the hinge for the conformational change that leaves the helices on the membrane surface. (Broken lines): hydrogen bond between $Gln^{111}$ and $Thr^{115}$. (**b**) Focus on the $Gln^{111}/Thr^{115}$ region showing the position and side-chain orientation of $Met^{115}$ in Cyt1A98 (A) and $Thr^{115}$ in Cyt1A97 (B). Broken lines are hydrogen bonds

C-D (Fig. 4b). Structural investigation of CytB and Cyt1Aa model shows without any doubt that $Gln^{111}$ ($Glu^{107}$ in CytB) is not the subject of any effect. This residue is positioned in the loop between $\beta 3$ and $\alpha C$, it is a solvent exposed residue, and it participates by its side chain to interact with water and with Ser113 ($Ser^{106}$ in CytB). Due to steric consideration, the position of $Gln^{111}$ cannot be altered, and then the interaction of the carbonyl group of $Gln^{111}$ with the hydroxyl group of $Thr^{115}$ may occur. This interaction is thought to be the reason of the inhibition of Cyt1A97 toxicity by disturbing C-D hinge motion. The loss of Cyt1A97 cytolytic activity was also confirmed by comparing the cytocidal activity, performed on HeLa cells culture, of the *B. thuringiensis israelensis* BUPM97 and BUPM98 parasporal crystals containing their respective Cyt proteins. The obtained results (data not shown) showed that BUPM98 solubilized crystals inhibited the HeLa cells growth but those of BUPM97 affected them weekly. These results demonstrate that Met at position 115 could play a key role in facilitating translocation of some parts of the protein through the membrane and is determinant for protein orientation and cytolytic activity on *E. coli.*

**Acknowledgements** We thank Mrs. Najeh Belguith-Ben Hassen for her technical assistance. This work was supported by grants from the Tunisian Ministère de l'Enseignement Supérieur de la Recherche Scientifique et de la Technologie et du développement des compétences.

## References

1. Promdonkoy, B., Chewawiwat, N., Tanapongpipat, S., Luxananil, P., & Panyim, S. (2003). Cloning and characterization of a cytolytic and mosquito larvicidal d-endotoxin from *Bacillus thuringiensis* subsp. *darmstadiensis*. *Current Microbiology, 46*, 94–98.
2. Cheong, H., & Gill, S. S. (1997). Cloning and characterization of a cytolytic and mosquitocidal d-endotoxin from *Bacillus thuringiensis* subsp. *jegathesan*. *Applied and Environmental Microbiology, 63*, 3254–3260.
3. Juarez-Perez, V., Guerchicoff, A., Rubinstein, C., & Delecluse, A. (2002). Characterization of Cyt2Bc toxin from *Bacillus thuringiensis* subsp. *medellin*. *Applied and Environmental Microbiology, 68*, 1228–1231.

4. Drobniewski, F. A., & Ellar, D. J. (1988) Investigation of the membrane lesion induced *in vitro* by two mosquitocidal delta-endotoxins of *Bacillus thuringiensis*. *Current Microbiology, 16*, 195–199.
5. Koni, P. A., & Ellar, D. J. (1994) Biochemical characterisation of *Bacillus thuringiensis* cytolytic delta-endotoxins. *Microbiology, 140*, 1869–1880.
6. Schnepf, H. E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., & Dean, D. H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews, 62*, 775–806.
7. Crickmore, N., Bone, E. J., Williams, J. A., & Ellar, D. J (1995). Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. *FEMS Microbiology Letters, 131*, 249–254.
8. Wu, D., & Federici, B. A. (1993) A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *Journal of Bacteriology, 175*, 5276–5280.
9. Ibarra, J., & Federici, B. A. (1986). Isolation of a relatively nontoxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. *Journal of Bacteriology, 165*, 527–533.
10. Wu, D., & Chang, F. N. (1985). Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal. *FEBS Letters, 190*, 232–236.
11. Georghiou, G. P., & Wirth, M. C. (1997). Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). *Applied and Environmental Microbiology, 63*, 1095–1101.
12. Pérez, C., Fernandez, L. E., Sun, J., Folch, J. L., Gill, S. S, Sorberon, M., & Bravo, A. (2005). *Bacillus thuringiensis* subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 18303–18308.
13. Wirth, M. C., & Georghiou, G. P. (1997). Cross-resistance among CryIV toxins of *Bacillus thuringiensis* subsp. *israelensis* in *Culex quinquefasciatus* (Diptera: Culicidae). *Journal of Economic Entomology, 90*, 1471–1477.
14. Wirth, M. C., Georghiou, G. P., & Federici, B. A. (1997). CytA enables CryIV endotoxins of *Bacillus thuringiensis* to overcome high levels of CryIV resistance in the mosquito, *Culex quinquefasciatus*. *Proceedings of the National Academy of Sciences of the United States of America, 94*, 10536–10540.
15. Douek, J., Einav, M., & Zaritsky, A. (1992). Sensitivity to plating of *Escherichia coli* cells expressing the *cytA* gene from *Bacillus thuringiensis* var. *israelensis*. *Molecular & General Genetics, 232*, 162–165.
16. Ballesta, J. P., Cundliffe, E., Daniels, M. J., Silverstein, J. L., Susskind, M. M., & Schaechter, M. (1972). Some unique properties of the deoxyribonucleic acid-bearing portion of the bacterial membrane. *Journal of Bacteriology, 112*, 195–199.
17. Thomas, W. E., & Ellar, D. J. (1983). *Bacillus thuringiensis* var. *israelensis* crystal delta- endotoxin: Effects on insect and mammalian cells *in vitro* and *in vivo*. *Journal of Cell Science, 60*, 181–197.
18. Promdonkoy, B., & Ellar, D. J. (2003). Investigation of the pore forming mechanism of a cytolytic d-endotoxin from *Bacillus thuringiensis*. *The Biochemical Journal, 374*, 255–259.
19. Manceva, S. D., Pusztai-Carey, M., Russo, P. S., & Butko, P. (2005). A detergent-like mechanism of action of the cytolytic toxin Cyt1A from *Bacillus thuringiensis* var. *israelensis*. *Biochemistry, 44*, 589–597.
20. Li, J., Koni, P. A., & Ellar, D. J. (1996). Structure of the mosquitocidal delta-endotoxin CytB from Bacillus *thuringiensis sp. kyushuensis* and implications for membrane pore formation. *Journal of Molecular Biology, 257*, 129–152.
21. Butko, P. (2003). Cytolytic toxin Cyt1A and its mechanism of membrane damage: Data and hypotheses. *Applied and Environmental Microbiology, 69*, 2415–2422.
22. Gazit, E., & Shai, Y. (1993). Structural characterization, membrane interaction, and specific assembly within phospholipids membranes of hydrophobic segments from *Bacillus thuringiensis* var. *israelensis* cytolytic toxin. *Biochemistry, 32*, 12363–12371.
23. Jaoua, S., Zouari, N., Tounsi, S., & Ellouz, R. (1996). Study of the δ-endotoxins produced by three recently isolated strains of *Bacillus thuringiensis*. *FEMS Microbiology Letters, 145*, 349–354.
24. Zghal, R. Z., & Jaoua, S. (2006). Evidence of DNA rearrangements in the 128-kilobase pBtoxis plasmid of *Bacillus thuringiensis israelensis*. *Molecular Biotechnology, 33*, 191–198.
25. Guzman, L. M., Belin, D., Carson, M. J., & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *Journal of Bacteriology, 177*, 4121–4130.
26. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor NY: Cold Spring Harbor Laboratory.
27. Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). The ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*, 4673–4680.
28. Schwede, T., Kopp, J., Guex, N., & Peitsch, M. C. (2003). SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Research, 31*, 3381–3385.
29. DeLano, W. L. (2002) The PyMOL Molecular Graphics System on World Wide Web http://www.pymol.org.
30. Chothia, C., & Lesk, A. M. (1986). The relation between the divergence of sequence and structure in proteins. *The EMBO Journal, 5*, 823–826.
31. Koni, P. A., & Ellar, D. J. (1993). Cloning and characterization of a novel *Bacillus thuringiensis* cytolytic delta-endotoxin. *Journal of Molecular Biology, 229*, 319–327.
32. Szabo, E., Murvai, J., Fabian, P., Fabian, F., Hollosi, M., Kajtar, J., Buzas, Z., Sajgo, M., Pongor, S., & Asboth, B. (1993). Is an amphiphilic region responsible for the haemolytic activity of *Bacillus thuringiensis* toxin? *International Journal of Peptide and Protein Research, 42*, 527–532.
33. Zghal, R. Z., Tounsi, S., & Jaoua, S. (2006). Characterization of a *cry4Ba*-type gene of *Bacillus thuringiensis israelensis* and evidence of the synergistic larvicidal activity of its encoded protein with Cry2A delta-endotoxin of *B. thuringiensis kurstaki* on *Culex pipiens*. *Biotechnology and Applied Biochemistry, 44*, 19–25.
34. Butko, P., Huang, F., Pusztai-Carey, M., & Surewicz, W. K. (1996) Membrane permeabilization induced by cytolytic delta-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis*. *Biochemistry, 35*, 11355–11360.