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Exposing cryptic antibacterial activity in Cyt1Ca from *Bacillus thuringiensis israelensis* by genetic manipulations

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Abstract In an attempt to endow Cyt1Ca with Cyt1Aa-like antibacterial activity, both derived from *Bacillus thuringiensis* subsp. *israelensis*, two amino acids were replaced, E117V and N125A, so as to raise the hydrophobicity of the corresponding region, considered to be the membrane-active motif. The clones obtained included multiple repeats of VIEVLKSLLGIALA, corresponding to head-to-tail polymerization of the primer, translated in frame with Cyt1Ca. These versions of Cyt1Ca caused instant arrest in biomass growth and decreased viability upon expression in *Escherichia coli*. Multiple insertions of the non-mutated motif VIEELKSLLGINLA into the polypeptide were also lethal. To expose toxicity of the latter motif in the original Cyt1Ca, *cyt1Ca* was appropriately truncated.

Keywords: Cytolytic proteins; Toxicity restoration; Multiple repeated motif; Microgene polymerization reaction

1. Introduction

The major component of the parasporal crystal of *Bacillus thuringiensis* subsp. *israelensis*, a gram-positive bacterium pathogenic for Dipteran species [1], is Cyt1Aa [2,3], which effectively lyses a broad range of cells and so belongs to the cytolytic family of *B. thuringiensis* toxins [4]. Despite low larvicidal activity by itself, Cyt1Aa contributes to the overall toxicity of *B. thuringiensis* by acting synergistically with the receptor-specific Cry toxins [5,6], and hence suppresses resistance to them in target insects [7–9]. Moreover, Cyt1Aa exerts strong toxicity on bacteria when produced internally [10–13] or applied externally [14].

A new coding sequence *cyt1Ca* has recently been detected on pBtoxis, the large toxin-coding plasmid of *B. thuringiensis* subsp. *israelensis* [15], with predicted product of a two-domain fusion protein. The N-terminal part is 52% identical to Cyt1Aa and the C-terminal part is similar to the receptor binding domain of ricin-B lectin type, found in several toxins: ricin, *Clostridium botulinum* neurotoxin and the mosquito-larvicidal Mtx1 from *B. sphaericus* [15]. Despite its high similarity to Cyt1Aa, neither full-length Cyt1Ca nor its Cyt1A-like truncated versions were bactericidal or synergistic to Cry4Aa against *Aedes aegypti* larvae [16]. No function has yet been assigned for it despite *cyt1Ca* transcription in *B. thuringiensis* [17].

Consecutive site-directed replacements of Q149K, Q159E, A185C and G235D according to Cyt1Aa revealed some Cyt1Ca variants with antibacterial but not larvicidal activities [18]. The acquired toxicities were attributed to stronger electrostatic interactions of the modified Cyt1Ca versions to lipid head groups in the bacterial inner membrane. The much lower antibacterial activities than those of Cyt1Aa are likely due to poor interactions of the mutated versions with the hydrophobic lipid core of the membrane. Cyt1Aa indeed includes one region of increased hydrophobicity (Fig. 1) that overlays on one of two α-helices that were proposed to play a significant role in the initial binding of the toxin to the membrane [19]. The hydrophobicity of α helix C and its adjacent regions of the Cyt1A-like domain of Cyt1Ca are still lower than that of Cyt1Aa (Fig. 1A). To raise it to a value similar to that of Cyt1Aa, E117 was replaced by V and N125 by A (Figs. 1B, 2A). The results uncover some cryptic bacteriocidity of Cyt1Ca.

2. Materials and methods

2.1. Bacterial strains and plasmids

All plasmids used in this study (Table 1) were introduced into *Escherichia coli* strain XL-Blue MRF'.

2.2. Site-directed mutagenesis

The method used was based on Stratagene's QuickChange Site-Directed Mutagenesis Kit. The recombinant plasmid pUH-*cyCtrC* (Table 1) was used as template for the double substitution E117V-N125A and *BspH*I site elimination, together with the primers Pr-E117V-N125A and Pr-cyCΔBspH (Table 2) and their respective complements. Generation of each mutation was confirmed by examining the corresponding restriction enzymes (Table 2), and sequencing.

2.3. Inserting products of microgene polymerisation reaction (MPR) [20] into cyt1Ca

MPR products were generated from primer Pr-MPR (Table 2) and its complement, which are related to the α-helix C of Cyt1Aa. A 50-μl reaction mixture contained 16 pmol of each of the primers, 4 μg of pUH-*cyCtrC* and 25 nmol of each of the dNTPs, in 10 mM KCl,10 mM $(NH_4)_2SO_4$, 20 mM Tris–HCl (pH 8.8), 8 mM $MgSO_4$, 0.1% Triton X-100, and 2 unit of Vent DNA polymerase (MBI Fermentas). The reaction started by pre-heating at 94.0 °C for 10 min, was followed by 65 cycles at 69.4 °C for 1 min and 72.2 °C for 1 min (these cycle conditions, lacking high temperature denaturation stage, were used to generate multiply repeated DNA from Pr-MPR homo-duplex without vector amplification), and finalized by 16 cycles at 94.0 °C for 30 s 50.0 °C for 1 min and 72.0 °C for 3 min (these cycles include high temperature vector denaturation stage for inserting the multiply repeated homo-duplex products into a specific site in the frame of *cyt1Ca*). The PCR product was treated by *Dpn*I overnight

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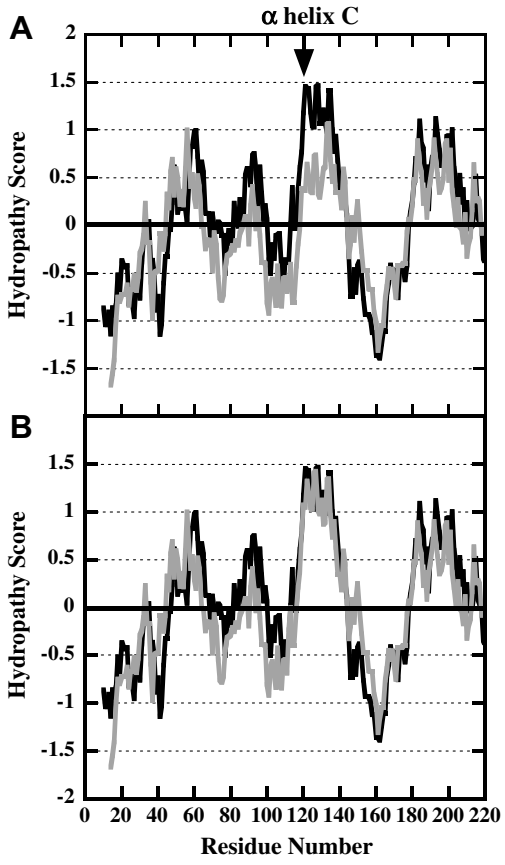

Fig. 1. Hydropathy score analyses of Cyt1Aa (black lines) and Cyt1Ca (grey lines), the latter truncated at N244, original version (A) and with E117V and N125A replacements (B). The arrow points to the position of the α helix C of Cyt1Aa.

and used to transform *E. coli* XL-Blue MRF'. Transformants were grown on LB plates with 100 μg ampicilin ml^{-1} and replicated onto the same plates with 1 mM IPTG. Clones which were non-viable on IPTG-containing plates were further studied.

2.4. Construction of plasmids with truncated versions of cyt1Ca

The primers Pr-pUH-Direct and Pr-cyC438C-Rev were employed to obtain a 3′ truncated *cyt1Ca* at position 438 (amplicon *cy*-α*Ctr* coding for Cyt1Ca truncated at Y146 (C-terminus)); Pr-cyC330N-Direct and Pr-pUH-Rev were employed to obtain a double truncated *cyt1Ca* at 330 (5′) and 732 (3′) (amplicon *cy*-α*Ntr* coding for Cyt1Ca truncated at M110 (N-terminus) and N244 (C-terminus)) (Tables 1 and 2; Fig. 2). These were PCR-amplified from pUH-*cyCtrC* and pUH-*cyCtrCΔBspH* as templates, respectively (Table 1) with Vent polymerase (MBI Fermentas) in a DNA thermal cycler for 30-reaction cycle each of 50 s at 94 °C, 50 s at 50 °C and 40 s at 72 °C. The 599-bp amplicon for *cy*-α*Ctr* was digested with *Xho*I/*Xba*I (Table 2) and inserted into the same sites of pUHE-24S to yield pUH-*cy*-α*Ctr*. The 449-bp amplicon for *cy*-α*Ntr* was digested with *Bsph*I/*Xba*I (Table 2) and inserted into*Nco*I/*Xba*I-cut pUHE-24S to yield pUH-*cy*-α*Ntr*.

2.5. Viable cell counts

Viability was determined by colony-forming ability (after 24 h incubation at 37 °C) on LB plates with 100 μg ampicillin ml^{-1} following appropriate dilutions.

2.6. Western blot analysis

E. coli cells were harvested by centrifugation 3–4 h after induction, re-suspended in distilled water, and boiled (10 min) in sample treatment buffer (62.5 mM Tris–Cl, pH 6.8, 2% SDS, 10% glycerol (v/v), 0.01% Bromophenol blue and 0.1 M DTT). Samples were analyzed by SDS–PAGE [23]. Proteins were electro-transferred from the gel onto nitrocellulose membranes and exposed to anti-Cyt1Ca antiserum (kindly provided by Dr. Colin Berry of Cardiff University, UK). Protein A-alkaline phosphatase conjugate was the primary antibody detector. Visualization of the antigen was achieved using Sigma Fast™-5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium.

2.7. Microscopy

Aliquots of growing cultures were fixed by 0.25% formaldehyde for morphology determinations of cells and inclusion bodies, whereas unfixed aliquots were used for determination of bacterial membrane

Table 1
PUHE-24S-based plasmids encoding the described products

Plasmid (amplicon) name	Description of product	Primers[a]	Ref.
pUHE-24S	Empty vector		[11]
pRM4-C (*cyt1Ca*)	Cyt1Aa		[11]
pUH-*cyCtrC* (*cyCtrC*)	Cyt1Ca truncated at N244 (CyCtrC) (Fig. 2)		[18]
pUH-*cyCtrCΔBspH* (*cyCtrCΔBspH*)	pUH-*cyCtrC* Quick-changed to eliminate *BspH*I site	Pr-cyCΔBspH and its complement	This study
pUH-*cy*-α*Ctr* (*cy*-α*Ctr*)	CyCtrC truncated at Y146 at C terminal (Fig. 2)	Pr-pUH-Direct and Pr-cyC438C-Rev	This study
pUH-*cy*-α*Ntr* (*cy*-α*Ntr*)	CyCtrC truncated at M110 at N terminal (Fig. 2)	Pr-cyC330N-Direct and Pr-pUH-Rev	This study
pUH-*cyCEVNA* (*cyCEVNA*)	CyCtrC with amino acid changes E117V N125A (Fig. 2)	Pr-E117V-N125A and its complement	This study
pUH-*cyCEVNA-2Rep* (*cyCEVNA-2Rep*)	CyCtrC containing 2 repeats of primer Pr-E117V-N125A (Fig. 3A)	Pr-E117V-N125A and its complement	This study
pUH-*cyCEVNA-2RepT* (*cyCEVNA-2RepT*)	CyCtrC containing 2 repeats of primer Pr-E117V-N125A with termination (Fig. 3C)	Pr-E117V-N125A and its complement	This study
pUH-*cyCEVNA-6Rep* (*cyCEVNA-6Rep*)	CyCtrC containing 6 repeats of primer Pr-E117V-N125A (Fig. 3B)	Pr-E117V-N125A and its complement	This study
pUH-*cy-2Rep* (*cy-2Rep*)	CyCtrC containing 2 repeats of primer Pr-MPR (Fig. 6B)	Pr-MPR and its complement	This study
pUH-*cy-3RepT* (*cy-3RepT*)	CyCtrC containing 3 repeats of primer Pr-MPR with termination (Fig. 6A)	Pr-MPR and its complement	This study

[a]Sequences of the primers are shown in Table 2.

Table 2
Sequences of the primers used (Table 1)

Primer	Sequence (5′–3′)[a]	Restriction enzyme
Pr-E117V-N125A	GGTGATAGAAG**T**GCTTAAATCTTTATTAGGAATT**GC**TCTGGC	*Vsp*I eliminated
Pr-cyCΔBspH	CGCAATTCAAGTTATGCAGCCATTGAT**C**CATGAGAGTTTTCAACCC	*BspH*I eliminated
Pr-pUH-Direct	CCCTTTCGTCTTCACCTCGAGAAAATTTATCAAAAAG	*Xho*I
Pr-cyC438C-Rev	CCAAAAAAGCCATCTAGATTGCTGTTGT**TA**GTAGAGG	*Xba*I
Pr-cyC330N-Direct	CAAGTACAGT**T**CATGATAAATAAGGTGATAGAAGAG	*Bsph*I generated
Pr-pUH-Rev	CGCGCGAGGCAGCTCTAGAGCACTTC	*Xba*I
Pr-MPR	GGTGATAGAAGA**A**CTTAAATCTTTATTAGGAATTAA**C**CTGGC	*Vsp*I eliminated

[a]Changed nucleotides are indicated in bold type; restriction enzyme recognition sites are underlined.

perforation. The samples were immobilized on object slides coated with solidified 1% solution of agarose [25], containing 0.9% NaCl (fixed samples) or LB broth and 0.5 μg ml^{-1} Ethidium Bromide (unfixed samples). Cells were visualized by phase-contrast or fluorescence (unfixed samples only) microscope (Nicon eclipse TE 2000-5; fluorescence filter: excitation at 540 and emission at 605 nm), equipped with Nicon sight DS-U1 CCD camera and photographed using NIS-Elements Br 2.10 software.

3. Results

The double mutant of the Cyt1A-like domain of Cyt1Ca, containing E117V-N125A, was non-viable on IPTG-containing plates. A repeated motif, VIEVLKSLLGIALA, was found in these clones in variable numbers (up to six), corresponding to head-to-tail tandem polymerization of the primer, sometimes accompanied by deletion of one or several nucleotides at the junctions between the primer units (Fig. 3). The variants thus produced encode a protein, either with an embedded repeated motif (Fig. 3A and B) or which exits frame at a certain repeat (Fig. 3C). This phenomenon, known as microgene polymerization reaction (MPR), will briefly be discussed below and detailed elsewhere (manuscript in preparation). The corresponding larger protein bands obtained by Western blot analysis with anti-Cyt1Ca antibodies (compare lanes 5 and 6 with lane 4 in Fig. 4) are consistent with the presence of repeats in the Cyt1Ca versions.

IPTG-induction of the repeats-including *cyt1Ca* variants slowed (in pUH-*cyCEVNA-2Rep*) or completely arrested (in

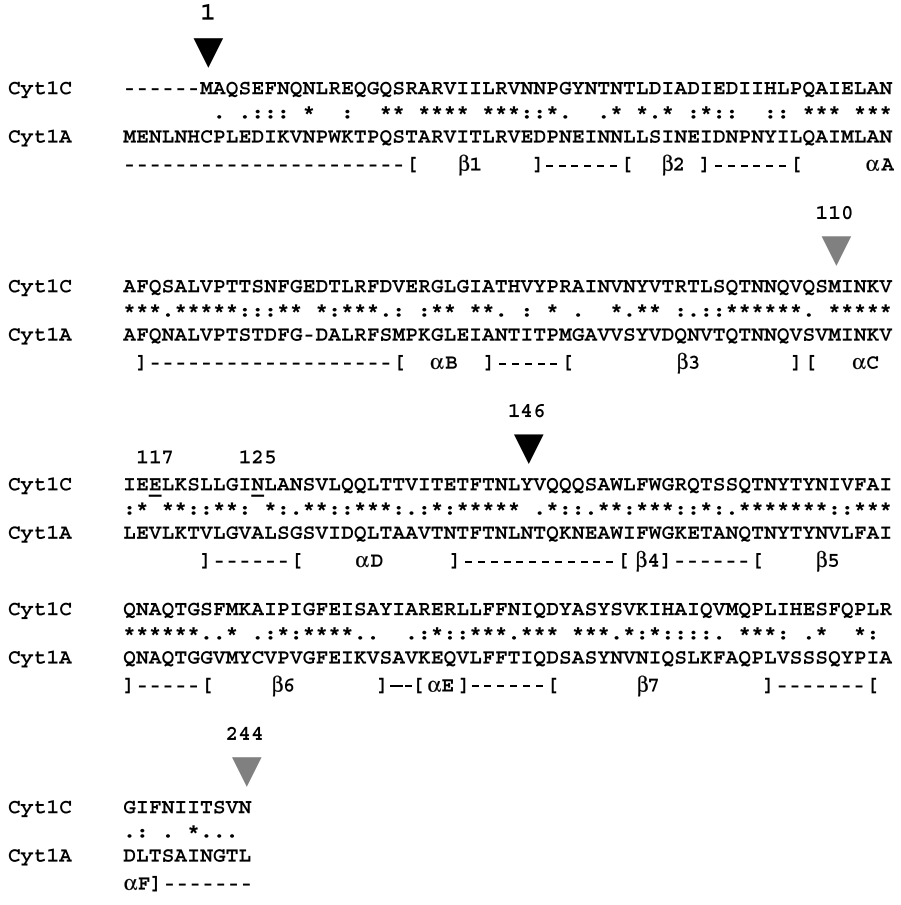

Fig. 2. Sequence alignment of Cyt1Aa and the Cyt1A-like domain of Cyt1Ca (clone pUH-*cyCtrC*) with the secondary structure elements in Cyt2Aa [24]. The amino acids replaced in this study are underlined and numbered. Arrowheads point to Cyt1Ca truncations to expose α helix C and D at the C- and N-termini (clones pUH-*cy*-α*Ctr* and pUH-*cy*-α*Ntr*) (black and grey, respectively).

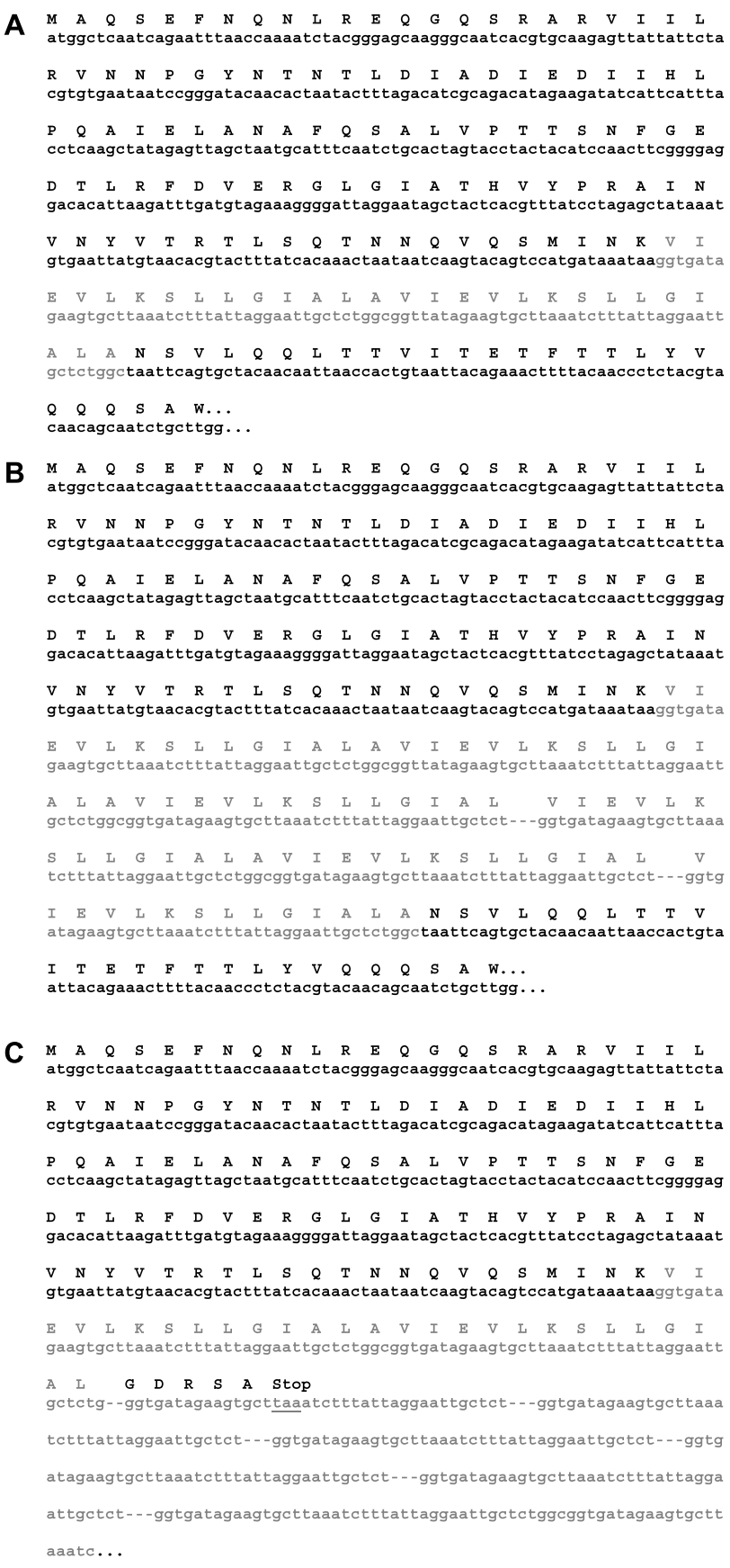

Fig. 3. Examples of encoding nucleotides and the corresponding amino acids sequences of Cyt1Ca variants toxic to *E. coli* in pUH-*cyCEVNA-2Rep* (A), pUH-*cyCEVNA-6Rep* (B) and pUH-*cyCEVNA-2RepT* (C). Dashes indicate deletions, and points indicate continued sequence as in Cyt1Ca C-terminally truncated at N244. The repeated region is shown in grey.

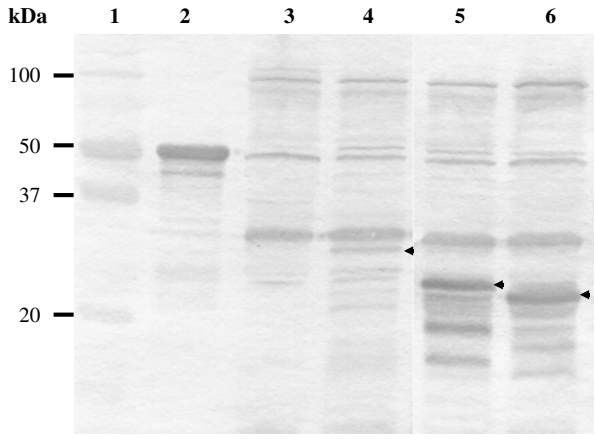

Fig. 4. Immunoblot analysis of mutated Cyt1Ca versions in clones pUH-*cyCEVNA-6Rep*, pUH-*cyCEVNA-2Rep* and pUH-*cyCEVNA* (lanes 4, 5 and 6, respectively). Lane 3, extract of a clone harboring empty vector. Lane 2, purified full-length His-tagged Cyt1Ca [16]. Lane 1, molecular size marker. Arrowheads point to bands, suggested for Cyt1Ca versions, contained in the corresponding clones.

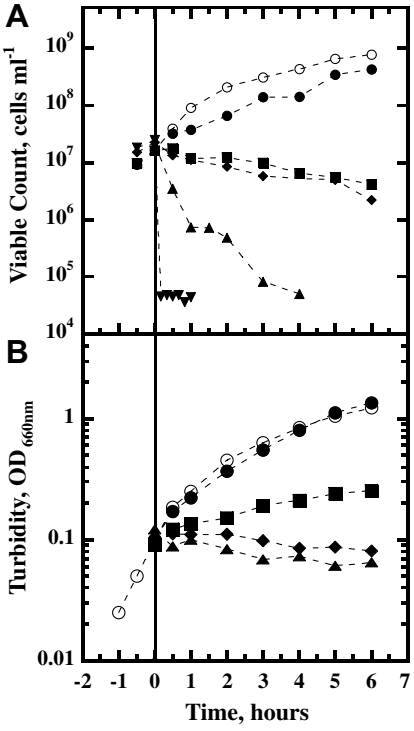

Fig. 5. Viable cell counts (A) and biomass (B) of *E. coli* clones, induced (closed symbols) with IPTG at time 0 (vertical line) and uninduced (open circles), harbouring either pUH-*cyCEVNA* (circles), pUH-*cyCEVNA-2Rep* (squares), pUH-*cyCEVNA-6Rep* (diamonds), pUH-*cyCEVNA-2RepT* (triangles), or pRM4-C (inverse triangles).

pUH-*cyCEVNA-6Rep* and pUH-*cyCEVNA-2RepT*) biomass growth (Table 1; Fig. 5B), accompanied by immediate decreased viability (Fig. 5A). A most dramatic decrease in viability after induction, approaching that of the clone expressing *cyt1Aa*, was displayed by clone pUH-*cyCEVNA-2RepT*. The toxicity revealed in the above clones may therefore be attributed to the motif repetition, either as such or with the mutated version. This is confirmed by the fact that induction of pUH-*cyCEVNA* (containing the E117V-N125A double mutation with no repeats) was not toxic to *E. coli* (Fig. 5).

To distinguish between these two possibilities, the repetitive non-mutated motif VIEELKSLLGINLA was inserted into the Cyt1A-like domain of Cyt1Ca as described in Section 2.3. All selected clones that were non-viable on IPTG-containing plates include head-to-tail primer repeats that are translated with this repeated oligopeptide motif, either embedded in Cyt1Ca frame or terminated early by an occasional non-sense codon (Fig. 6). Two of these versions, pUH-*cy-2Rep* and pUH-*cy-3RepT* (Table 1), respectively, were toxic to their *E. coli* host, indicating a crucial role of the repetitive motif in the lethal action (Fig. 7).

The observed lethality to *E. coli* of these Cyt1Ca derivatives indicates that the motif of Cyt1Ca homologous to fragment of α helix C and its downstream inter-helical loop of Cyt1Aa are responsible. In order to expose the presumed hidden toxicity attributed to this helix in the original, not active Cyt1A-like domain of Cyt1Ca (clone pUH-*cyCtrC* (Table 1)), two truncated versions were generated (Fig. 2), the considered motif in one is situated near the N terminus of the protein whereas in the second, it is situated near the C terminus. Indeed, induction of these truncated versions instantly arrested both biomass growth and viability in a way similar to clones containing the multiply repeated motifs described above (Fig. 8).

4. Discussion

Certain previously derived mutational changes endow the Cyt1A-like domain of Cyt1Ca with antibacterial effects [18]. These effects were however weak compared with those of Cyt1Aa: no arrest in biomass growth was observed after induction of the mutated versions, viability decreased slightly at best after a lag of 1 h, and cell divisions resumed after some time. In this study, the cryptic *cyt1Ca* was further modified aiming to reveal its potential function in *B. thuringiensis* subsp. *israelensis*. The mutational manipulations were directed to the sequence homologous to the α helix C and its downstream inter-helical loop of Cyt1Aa (Fig. 1) because this sequence had been proposed as the region important for Cyt1Aa activity [19].

A MAQSEFNQNLREQGQSRARVIILRVNNPGYNTNTLDIADIEDIIHLPQAIELANAFQSAL
VPTTSNFGEDTLRFDVERGLGIATHVYPRAINVNYVTRTLSQTNNQVQSMINKVIEELKS
LLGINL--IEELKSLLGINL-VIEELKSLLGINL stop

B MAQSEFNQNLREQGQSRARVIILRVNNPGYNTNTLDIADIEDIIHLPQAIELANAFQSAL
VPTTSNFGEDTLRFDVERGLGIATHVYPRAINVNYVTRTLSQTNNQVQSMINKVIEELKS
LLGINL--IEELKSLLGINLANSVLQQLTTVITETFTNLYVQQQSAW...

Fig. 6. Amino acid sequences of Cyt1Ca versions toxic to *E. coli* produced in the clones: pUH- *cy-3RepT* (A) and pUH-*cy-2Rep* (B). Dashes indicate deletions, and points indicate continued sequence as in Cyt1Ca C-terminally truncated at N244. The repeated regions are shown in grey.

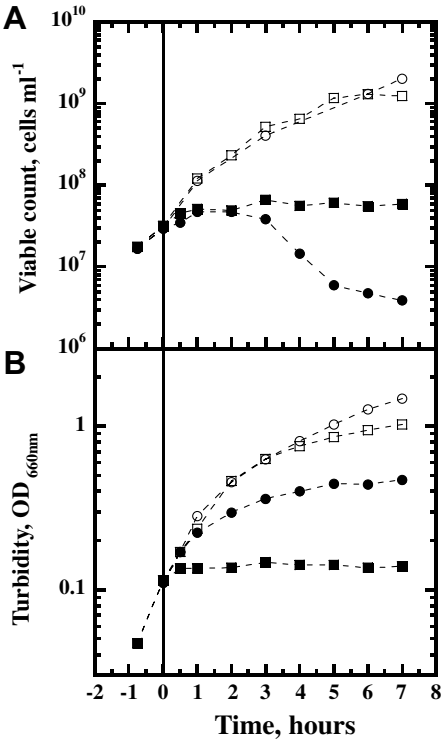

Fig. 7. Viable cell counts (A) and biomass (B) of *E. coli* clones, induced (closed symbols) with IPTG at time 0 (vertical line) and uninduced (open symbols), harbouring either pUH-*cy-2Rep* (circles) or pUH-*cy-3RepT* (squares).

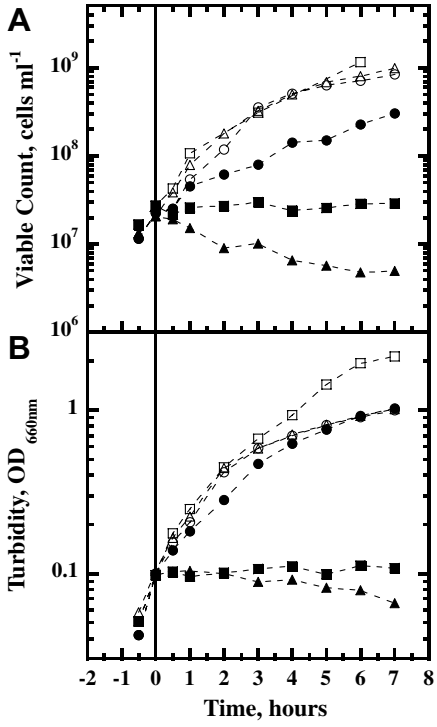

Fig. 8. Viable cell counts (A) and biomass (B) of *E. coli* clones, induced (closed symbols) with IPTG at time 0 (vertical line) and uninduced (open symbols), harbouring pUH-*cy*-α*Ctr* (squares), pUH-*cy*-α*Ntr* (triangles), pUH-*cyCtrC* (circles).

Biomass growth was arrested immediately after induction of all clones that produce Cyt1Ca variants terminated downstream α helix C-like motif (pUH-*cy-3RepT*, pUH-*cyCEVNA-2RepT*, pUH- *cy*-α*Ctr*, pUH-*cy*-α*Ntr*) and of one that is not terminated but contains six repeats of the above motif (pUH- *cyCEVNA-6Rep*) (Figs. 5, 7 and 8), indicating high toxicity of these derivatives to the host cell. Very high toxicity of pUH- *cyCEVNA-2RepT* (Fig. 5) may be attributed to probable membrane perforation by the corresponding protein derivative, similar to Cyt1Aa-producing clone [12], as can be assayed by Ethidium Bromide staining (Fig. 9). The other Cyt1Ca versions constructed here seem to not perforate the

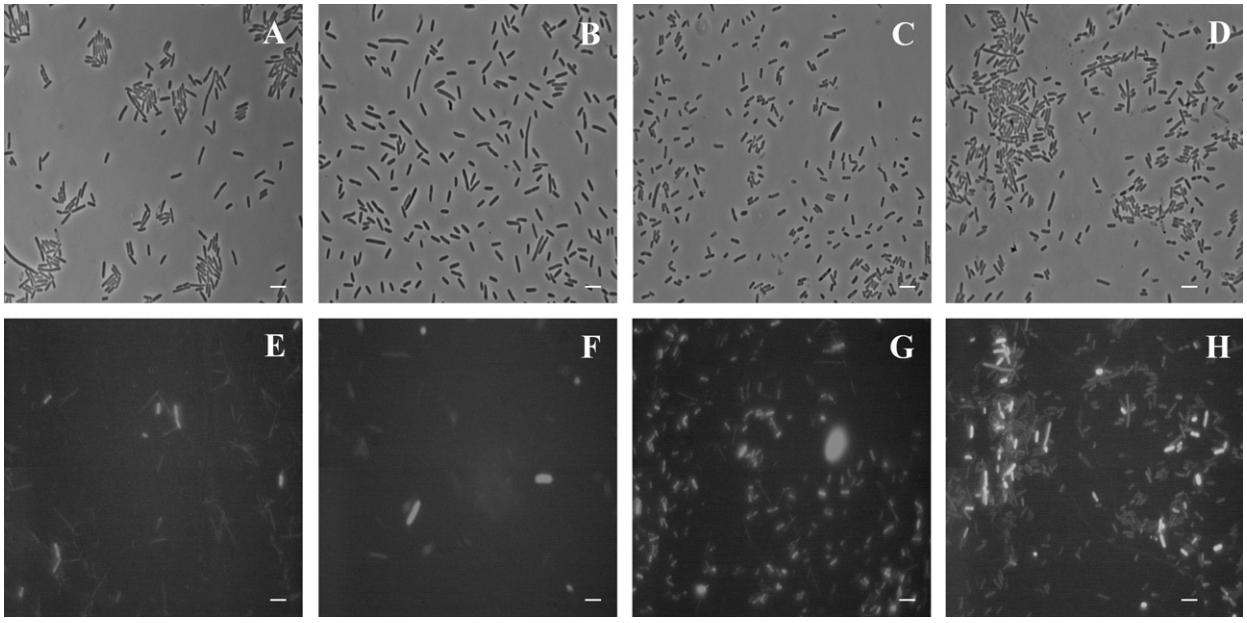

Fig. 9. Phase contrast (A–D) and fluorescence micrographs of ethidium bromide-treated (E–H) *E. coli* cells harboring plasmids pUHE-24S (A,E), pUH-*cy*-α*Ctr* (B,F), pRM4-C (C,G), pUH-*cyCEVNA-2RepT* (D,H), after 2 h induction. Magnification bars, 5 μm.

membrane (shown (Fig. 9) only for clone pUH- *cy*-αCtr) probably due to their milder effect on the bacterial inner membrane. As a consequence of blocking protein synthesis, these clones are unable to accumulate the corresponding proteins in sufficient amounts for good visualization by Western blot (data shown (Fig. 4) only for pUH-*cyCEVNA-6Rep*).

Inducing pUH-*cyCEVNA-2Rep* (Fig. 5B) or pUH-*cy-2Rep* (Fig. 7B) did not immediately arrest biomass growth. However, it was slowed down and accompanied with decreased viability. Only these clones produced the corresponding proteins aggregated into *multiple inclusion bodies* clearly seen after induction (Fig. 10, top panels J and I, respectively). The inclusion bodies seen on envelope of cells carrying pUH-*cyCEVNA-2Rep* (Fig. 10, top panels E and J) support our contention that the manipulated Cyt1Ca versions interact with the bacterial membrane [18]. Biomass growth of pUH-*cyCtrC* (Fig. 8B) and pUH-*cyCEVNA* (Fig. 5B) continued unimpeded and was accompanied by generation of *single inclusion body* at the late stage of induction (Fig. 10, top panels G and H, respectively). The higher toxicities of pUH-*cyCEVNA-2Rep* and pUH-*cy-2Rep* than those of pUH-*cyCtrC* and pUH-*cyCEVNA* may be explained by processing of the corresponding proteins into their toxic derivatives in the former two clones. This interpretation is consistent with the presence of low-molecular weight products detected by Western Blot analysis in pUH-*cyCEVNA-2Rep* and pUH-*cy-2Rep*, contrary to pUH -*cyCtrC* and pUH-*cyCEVNA* (Fig. 10, bottom panel). The amounts of these putatively toxic low-molecular weight products exceeded those of the truncated products mentioned in the preceding paragraph (hardly seen on the immunoblot) (data not shown) so their deleterious effect on the cells may be more pronounced. This may explain the more significant drop in viability in pUH-*cy-2Rep* than of pUH-*cy-3RepT* (Fig. 7A).

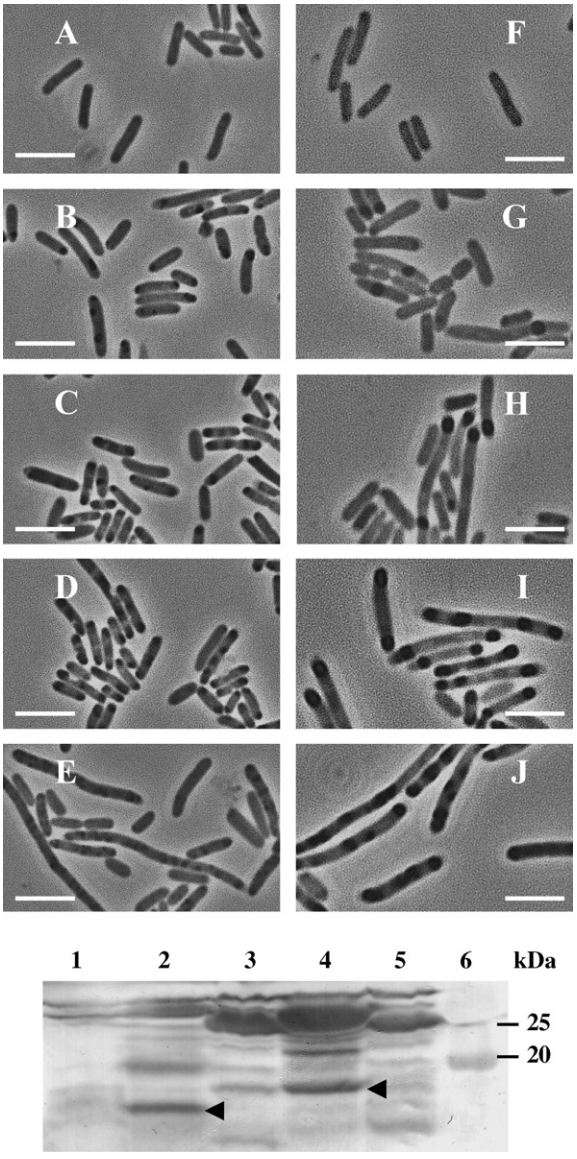

Fig. 10. Top. Phase contrast micrographs of *E. coli* cells harboring pUHE-24S (A,F), pUH-*cyCtrC* (B,G), pUH-*cyCEVNA* (C,H), pUH-*cy-2Rep* (D,I) and pUH-*cyCEVNA-2Rep* (E,J) after 2 (A–E) and 6 (F–J) hours of IPTG induction. Magnification bars, 5 μm. Bottom. Immunoblot analysis of mutated Cyt1Ca versions produced in the same clones: pUHE-24S (lane 1), pUH-*cyCEVNA-2Rep* (lane 2), pUH-*cyCEVNA* (lane 3), pUH-*cy-2Rep* (lane 4), pUH-*cyCtrC* (lane 5), molecular size marker (lane 6). Arrowheads point to bands, suggested for toxic derivatives of processed Cyt1Ca versions in the corresponding clones.

Different extents of toxicity of Cyt1Ca derivatives to host cells studied here may mean different extents of interplay between two processes involving the produced polypeptide: interaction with the bacterial membrane, and competitive self aggregation into inclusion body. Strong, stable interactions with the membrane of the highly toxic Cyt1Ca derivatives (clones pUH-*cy-3RepT*, pUH-*cyCEVNA-2RepT*, pUH-*cyCEVNA-6Rep*, pUH-*cy*-αCtr, pUH-*cy*-αNtr) may thus prevent their self-aggregation, whereas in the non-toxic strains (clones pUH-*cyCtrC* and pUH-*cyCEVNA*), they do not interact with the membrane, and not being tolerated as soluble proteins in the cytoplasm, they deposit around single aggregation nuclei forming single inclusion bodies. In moderately toxic strains (clones pUH-*cyCEVNA-2Rep* and pUH-*cy-2Rep*), protein–membrane interactions of Cyt1Ca derivatives is presumably weak, and hence their dynamic association/dissociation with/from the membrane allows some protein aggregation in close proximity to the membrane binding sites, the process which generates multiple inclusion bodies spread near the cell envelope.

Deriving repetitions of the non-mutated motif VIEELKSLLGINLA in Cyt1Ca was not straightforward. A special procedure was devised based on the head-to-tail primer polymerization, which sheds some additional light on the importance of this region to Cyt1Ca activity.

Head-to-tail primer polymerization, named microgene polymerization reaction (MPR), can result from intrinsic ability of a given pair of complementary oligonucleotides to expand into multiply repeated products without any template [20]. A detailed investigation of MPR with such oligonucleotides is described elsewhere (“The Microgene Polymerization Reaction”, Itsko et al., in preparation). Essentially, the method enhances expansion of the duplex into DNA repeats by cycling around its melting temperature [21]. This expansion is limited to the duplex, whereas inserting its oligopeptide-encoded product in frame with the gene in the vector (that needs high temperature for strand separation) is accomplished by additional standard PCR cycles, as detailed in Section 2.3. This phenomenon can actually be used for inserting relatively short peptide multiple motifs in frame with a protein.

The results demonstrate that the original motif of Cyt1Ca, VIEELKSLLGINLA, exerts deleterious effects on *E. coli* to an extent dependent on its surrounding amino acids. The toxicity is possibly hidden by other parts of Cyt1Ca due to its inherent folding. If so, nearby multiplication of this motif or translational termination may exert its toxic action by direct exposure or after some protease activation (Fig. 10, bottom panel). In order to detach this region from the other parts of Cyt1Ca and consequently prevent their interference to its antibacterial activities, the protein was truncated so that α helices C and D were at the N or C terminus (Fig. 2). The toxicity of these variants to *E. coli* (Fig. 8) supports this suggestion. The lower hydrophobicity of Cyt1Ca's α helix C than that of Cyt1Aa (Fig. 1) directs attention to some possible specific rather than just hydrophobic interaction(s) between it and cell cytoplasmic membrane, depending on specific pattern of amino acid side chains.

A biological function for Cyt1Ca is still to be discovered. The bactericidal character of the truncated versions *cy-αCtr* and *cy-αNtr* constructed here may point to possible proteolytic activation that it must undergo *in vivo* to expose its toxicity to some yet unknown target organism. The cryptic antibacterial action of the C-terminal domain of Cry1Ab is similarly hidden when produced in contiguity with the N-terminal insecticidal part of the toxin [22]. Sequestration of antibacterial activity of Cyt1Ca in a specific fold of the whole protein may provide a safety mechanism to protect the host bacterium.

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