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Partial Restoration of Antibacterial Activity of the Protein Encoded by a Cryptic Open Reading Frame (*cyt1Ca*) from *Bacillus thuringiensis* subsp. *israelensis* by Site-Directed Mutagenesis

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Insecticidal crystal proteins of *Bacillus thuringiensis* belong to two unrelated toxin families: receptor-specific Cry toxins against insects and Cyt toxins that lyse a broad range of cells, including bacteria, via direct binding to phospholipids. A new *cyt*-like open reading frame (*cyt1Ca*) encoding a 60-kDa protein, has recently been discovered (C. Berry et al., Appl. Environ. Microbiol. 68:5082–5095, 2002). Cyt1Ca displays the structure of a two-domain fusion protein: the N-terminal moiety resembles the full-length Cyt toxins, and the C-terminal moiety is similar to the receptor-binding domains of several ricin-like toxins, such as Mtx1. Neither the larvicidal activity of *cyt1Ca* expressed in *Escherichia coli* nor the hemolytic effect of His-tagged purified Cyt1Ca has been observed (R. Manasherob et al., unpublished). This was attributed to five amino acid differences between the sequences of its N-terminal moiety and Cyt1Aa. The 3′ end of *cyt1Ca* was truncated (removing the ricin-binding domain of Cyt1Ca), and six single bases were appropriately changed by site-directed mutagenesis, sequentially replacing the noncharged amino acids by charged ones, according to Cyt1Aa, to form several versions. Expression of these mutated *cyt1Ca* versions caused loss of the colony-forming ability of the corresponding *E. coli* cells to different extents compared with the original gene. In some mutants this antibacterial effect was associated by significant distortion of cell morphology and in others by generation of multiple inclusion bodies spread along the cell envelope. The described deleterious effects of mutated *cyt1Ca* versions against *E. coli* may reflect an evolutionary relationship between Cyt1Aa and Cyt1Ca.

Bacillus thuringiensis is a gram-positive soil bacterium that during sporulation produces large amounts of specific insect larvicidal proteins (known as δ-endotoxins) aggregated in parasporal bodies (6). It is widely used as a base for commercial preparations of microbial insecticides (27, 28, 34).

Insecticidal crystal proteins of different *B. thuringiensis* subspecies are classified into two unrelated families: receptor-specific Cry toxins that permeabilize the membrane of midgut insect cells and Cyt toxins that lyse a broad range of cells via direct binding to phospholipids (10).

Toxins of the Cyt family are particularly found in subspecies of *B. thuringiensis* that are toxic for Diptera (e.g., *B. thuringiensis* subsp. *israelensis*, *kyushuensis*, *medellin*, and *jegatesan*). These are relatively small proteins with molecular masses of around 25 to 28 kDa. Their cytolytic activities are attributed to the ability to bind phospholipids with unsaturated chains at the *syn-2* position (17, 32) that predominantly compose dipteran cell membranes (14).

Seven cytolytic, mosquitocidal toxins are currently known and characterized on the biochemical level (7, 12, 13, 19, 21, 31, 42); the most investigated toxin is Cyt1Aa from *B. thuringiensis* subsp. *israelensis*. Although Cyt1Aa exhibits low toxicity by itself, it acts highly synergistically with Cry toxins (9, 20) and heterologous toxins, such as Bin from *Bacillus sphaericus* (38). Because Cyt1Aa plays a critical role in suppressing resistance to Cry toxins in target insects (15, 39, 40), Cyt1Aa is invaluable for improving mosquito-controlling bacterial strains (16).

General cytolytic effects of Cyt toxins against a variety of eukaryotic (33) and prokaryotic cells attract interest beyond insect pest management. Antibacterial activity of Cyt1Aa is well reported: e.g., it is bactericidal to *Micrococcus luteus* (43). Expression of *cyt1Aa* in *B. thuringiensis* subsp. *kurstaki* (41) or *Escherichia coli* (11, 24) causes loss of viability, accompanied in the latter by pronounced nucleoid compaction (25). The cytotoxicity of Cyt1Aa against eukaryotic cells may render it useful for cancer treatment if specificity to tumor cells is raised by, for example, linking it chemically to targeting ligands (2). Due to potential important biological role for Cyt toxins, screening for new *cyt* genes is on the current agenda (18).

A new *cyt*-like open reading frame, *cyt1Ca*, encoding a 60-kDa protein, has recently been discovered in *B. thuringiensis* subsp. *israelensis* (5). Its predicted product displays the structure of a two-domain fusion protein: the N-terminal part resembles the common Cyt toxins, and the C-terminal part is similar to the receptor-binding domain of ricin-B lectin type, found in several unrelated toxins, such as ricin, *Clostridium botulinum* neurotoxin and the mosquito-larvicidal Mtx1 toxin from *B. sphaericus* (5).

Discovering a potentially cytolytic protein from the Cyt family with a binding domain raises an attractive hypothesis, that it is targeted to the cell via a receptor, as Cry family toxins do. This mode of action is rather new for Cyt toxins. The Cyt-like domain of Cyt1Ca is 72% homologous to Cyt1Aa. Neither the larvicidal activity of *cyt1Ca* expressed in *E. coli* nor the hemolytic effect of His-tagged purified Cyt1Ca was found (23). This

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A

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                                      ↓
Cyt1C   ------MAQSEFNQNLREQGQSRARVIILRVNNPGYNTNTLDIADIEDIIHLPQAIELAN
              . .::: *  :  ** **** ***::*.  .* *.* :*::  :: *** ***
Cyt1A   MENLNHCPLEDIKVNPWKTPQSTARVITLRVEDPNEINNLLSINEIDNPNYILQAIMLAN
        ---------------------[    β1    ]------[  β2 ]------[     αA

Cyt1C   AFQSALVPTTSNFGEDTLRFDVERGLGIATHVYPRAINVNYVTRTLSQTNNQVQSMINKV
        ***.*****:::** *:***.: :** **. : * .  *.** :.::******. *****
Cyt1A   AFQNALVPTSTDFG-DALRFSMPKGLEIANTITPMGAVVSYVDQNVTQTNNQVSVMINKV
         ]-------------------[  αB  ]-----[          β3        ][  αC

                                            149       159
Cyt1C   IEELKSLLGINLANSVLQQLTTVITETFTNLYVQQQSAWLFWGRQTSSQTNYTYNIVFAI
        :* **::**: *:.**::***:.:*:***** .*::.**:***::*:.*******::***
Cyt1A   LEVLKTVLGVALSGSVIDQLTAAVTNTFTNLNTQKNEAWIFWGKETANQTNYTYNVLFAI
            ]------[    αD     ]------------[ β4]------[     β5

               185                                  220        ↓
Cyt1C   QNAQTGSFMKAIPIGFEISAYIARERLLFFNIQDYASYSVKIHAIQVMQPLIHESFQPLR
        ******..* .:*:****..  .:*::***.*** ***.*:*::::. ***: .*  *:
Cyt1A   QNAQTGGVMYCVPVGFEIKVSAVKEQVLFFTIQDSASYNVNIQSLKFAQPLVSSSQYPIA
        ]-----[      β6      ]--[αE]------[      β7         ]-------[

                  ↓
        235
Cyt1C   GIFNIITSVNNRSAIQITEYYDENTHDYPVKLWDYNNIINQKWILVFNQTTRAYSIQNLI
        .:  . *...
Cyt1A   DLTSAINGTL--------------------------------------------------
        αF]-------
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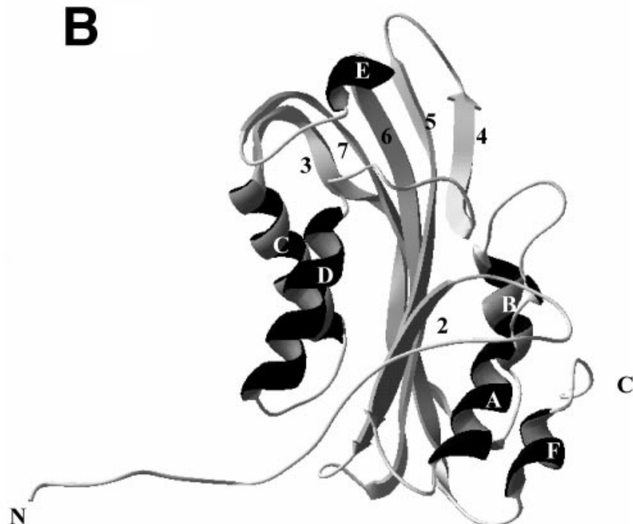

FIG. 1. (A) Sequence alignment of Cyt1Aa and the Cyt-like domain of Cyt1Ca with the secondary structure elements in Cyt2Aa. The amino acids important for Cyt1Aa activity (37) are shown in large type; the amino acids replaced in this study are shown in large grey type and numbered. Underlined amino acids are predicted to be on the Cyt1Aa surface (22, 26). Arrows point to Cyt1Ca truncations, according to the full-length Cyt1Aa (grey) and proteinase K activation (black). Gaps introduced to maximize alignment (dashes) are shown. (B) A schematic ribbon diagram determined by X-ray crystallography of a monomer of Cyt2Aa (22). The structure shows a three-layered α/β architecture where the α-helices form the outer layer and β-strands (marked as in reference 22) are buried within the core of the protein.

inactivity may be attributed to differences in five amino acids between its Cyt-like (N-terminal) moiety and Cyt1Aa (37) (Fig. 1): five noncharged amino acids (three Gln, a Gly, and an Ala) in the former compared to charged ones (two Lys, a Glu, and an Asp) and a Cys in the latter. In attempts to obtain a toxic variant(s) of Cyt1Ca and dissect the dual actions of Cyt1Aa, the 3′ end of *cyt1Ca* was truncated (removing the C-terminal domain), and thoroughly chosen single bases in the remaining domain were treated appropriately by site-directed mutagenesis to change the noncharged amino acids to charged amino acids or cysteine as in Cyt1Aa.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* XL-Blue MRF′ and plasmids used in this study are listed in Table 1.

Cultures were grown at 37°C in LB broth supplemented with 100 μg ml^{-1}

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TABLE 1. pUHE-24S-based plasmids encoding the described products

Plasmid	Description of product	Reference
pUHE-24S	Empty vector	24
pHE4-A	Cry4Aa	4
pRM4-C	Cyt1Aa	24
pUH-*cyCtrC*	Cyt1Ca truncated at N244 (CyCtrC)	This study
pUH-*cyCtrC12*	CyCtrC with amino acid changes Q149K Q159E	This study
pUH-*cyCtrC3*	CyCtrC with amino acid changes Q149K Q159E G235D	This study
pUH-*cyCtrC4*	CyCtrC with amino acid changes Q149K Q159E G235D A185C	This study
pUH-*cyCtrC5*	CyCtrC with amino acid changes Q149K Q159E G235D A185C Q220K	This study
pUH-*cyCtrC6*	CyCtrC with amino acid changes Q149K Q159E Q220K	This study
pUH-*cyCtrNC*	Cyt1Ca truncated at I21 and E228 (CyCtrNC)	This study
pUH-*cyCtrNC12*	CyCtrNC with amino acid changes Q149K Q159E	This study
pUH-*cyCtrNC6*	CyCtrNC with amino acid changes Q149K Q159E Q220K	This study
pUH-*cyCtrNC4*	CyCtrNC with amino acid changes Q149K Q159E A185C	This study

ampicillin to an optical density at 660 nm of 0.2 to 0.3 ($\approx 2 \times 10^8$ cells ml^{-1}) and induced by 1 mM of isopropyl-β-D-thiogalactoside (IPTG).

PCR. Truncated versions of *cyt1Ca* were amplified from pBtoxis of *B. thuringiensis* subsp. *israelensis* (5) with Taq polymerase (MBI Fermentas) in a DNA thermal cycler for 30 reaction cycles, with each cycle consisting of 50 s at 94°C, 50 s at 50°C, and 50 s at 72°C.

The primers for the C-terminally truncated version of Cyt1Ca (CyCtrC) were a 39-mer, 5′-CAGTAATT**TCTAGA**GCACTTCTTTAATTTACGGAAGTTA-3′, containing an XbaI restriction site (bold type) and a translation termination site (underlined), and a 30-mer, 5′-CCAGGGGGCGAGT**CCATGG**CTCAATCAGAA-3′, containing an NcoI restriction site (bold type). For the N- and C-terminally truncated versions (CyCtrNC), the primers were a 42-mer, 5′-CGTGCAAGAG**CCATGG**TTCTACGTGTGGAAGACCCGGGATAC-3′, containing an NcoI restriction site (bold type), and a 37-mer, 5′-GCCTCTTAGG**TCTAGA**AATTACTCATGAATCAATGGC-3′, containing an XbaI restriction site (bold type) and a translation termination site (underlined).

Construction of plasmids. The blunt-end PCR products (659- and 774-bp fragments for *cyCtrNC* and *cyCtrC*, respectively) were digested with NcoI/XbaI and inserted into the same sites of pUHE-24S to get pUH-*cyCtrC* and pUH-*cyCtrNC*, respectively.

Site-directed mutagenesis. The site-directed mutagenesis method used was based on Stratagene's QuikChange site-directed mutagenesis kit. The recombinant plasmids pUH-*cyCtrC* and pUH-*cyCtrNC* were used as templates for the single and double amino acid changes Q148K Q159E, G235D, A185C, and Q220K, together with the appropriate primers shown in Table 2. For multiple amino acid changes at distant places, templates of previously mutated versions were used. For each mutant, the recombinant plasmid was confirmed by digestion with the restriction endonuclease (Table 2), and the DNA sequence was verified by an automated DNA sequencer.

Viable cell counts. Cell viability was determined by measuring the ability to form colonies on LB plates (with 100 μg ampicillin ml^{-1}) following appropriate dilutions and with IPTG when indicated. The number of colonies was counted after 24 h of incubation at 37°C.

Microscopy. Aliquots of growing cultures were fixed (0.25% formaldehyde), immobilized on agarose slides as described previously (35), and cells were visualized by phase-contrast microscopy (Zeiss Axioplan 2) and photographed using IPLAB 3.1a (Signal Analytics).

Mosquito larvicidal assay. Cells were harvested by centrifugation after 4 h of induction and resuspended in distilled water. Samples were added to 20 early third-instar *Aedes aegypti* larvae in disposable cups with 100 ml sterile tap water, and mortality was determined after 24 h at 28°C (4). Presumed synergy between mutated versions of Cyt1Ca and Cry4Aa was tested using mixtures of two clones containing 4:1 ratios (by cell number) of clones expressing different *cyt1Ca* versions and a clone expressing *cry4Aa*, respectively.

RESULTS

Rationale for the selection of amino acids to be replaced in Cyt1Ca. Cyt1Aa has 13 positively and negatively charged amino acids that are critical for its activity; changing either of them to alanine affects its toxicity considerably (37). Aligning the sequence of the Cyt-like domain of Cyt1Ca with that of Cyt1Aa reveals that some of these 13 have changed to non-charged amino acids: K154 in Cyt1Aa changed to Q149 in Cyt1Ca; E164 in Cyt1Aa changed to Q159 in Cyt1Ca; K225 changed to Q220, and D240 changed to G235 (Fig. 1). Moreover, the only cysteine in the activated form of Cyt1Aa (C190) that is also important for its activity (8) is changed to alanine in Cyt1Ca (A185). The inactivity of the latter (23) is attributed to these five changes and hence we decided to sequentially change them back to those in the original Cyt1Aa. The replacements were implemented on two different truncated versions of Cyt1Ca: one (*cyCtrC*), at N244 (as in the full-length Cyt1Aa), and the second (*cyCtrNC*), at both ends, at I21 and E228 (as in proteinase K-activated fragment of Cyt1Aa). In addition, N6 and N7 in *cyCtrNC* were replaced by E6 and D7, respectively (as in Cyt1Aa).

Deleterious effects to *E. coli* of the mutated Cyt1Ca (Fig. 2). Expression of the nonmutated *cyCtrC* delayed cell division for an hour starting 30 min after induction. Its doubly mutated Q149K Q159E version (*cyCtrC12*) delayed cell division for 210 min. The additional mutations G235D and A185C (in *cyCtrC3* and *cyCtrC4*) emphasized this effect, leading to three- and sixfold drops in the colony-forming ability 3 h after induction, respectively. Very slow recoveries were observed afterwards. The last sequential mutation Q220K (in *cyCtrC5*) abolished the

TABLE 2. Sequences of the primers used to construct the site-directed mutants

Amino acid change	Primer sequence (5′-3′)[a]	Restriction enzyme
Q148K Q159E	CGTACAA**A**AGCAATCTGCTTGGCTTTTTTGGGGACGC**G**AAACATC	HinII eliminated
G235D	GAGAGTTTTCAACC**CTTAAG**AG**A**CATATTTAATATCATAACTTCCG	AflII
A185C	CAGGTAGTTTTATGAAA**T**GTATTCCTATAGGGTTTGAGAT**C**TCTGC	BglII
Q220K	CGCAATT**A**AAGTTATGCAGCCATTGATCCATGAGAGTTTTCAACCC	BspHI eliminated

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

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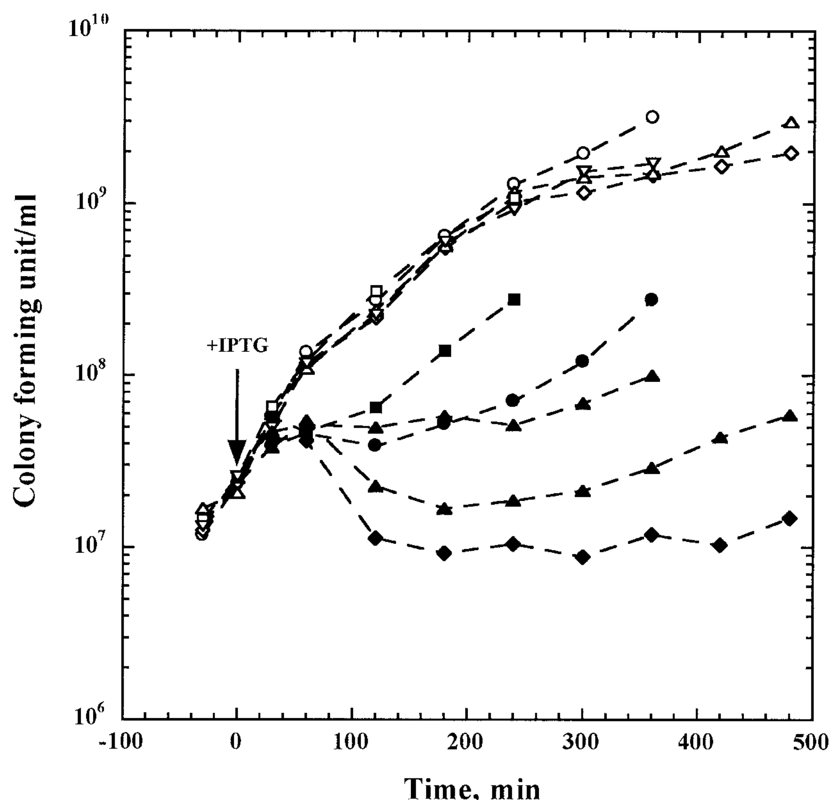

FIG. 2. Viable cell counts of *E. coli* strains harboring plasmids cloned with different *cyt1Ca* versions without (open symbols) or with (closed symbols) induction by IPTG: ■, *cyCtrC*; ●, *cyCtrC12*; ▲, *cyCtrC3*; ◆, *cyCtrC4*; ▼, *cyCtrC5*.

killing effect of *cyCtrC3* and *cyCtrC4* while division arrest remained.

The difference in the viability of cells expressing different mutated versions of the truncated *cyt1Ca* was emphasized upon indefinite induction, when cells were spread out on IPTG-containing plates (Table 3). Here, viabilities of cells with both truncated versions were monitored and compared with that of the clone expressing *cyt1Aa*. Surprisingly, the mutant *cyCtrNC4* (with four substitutions, i.e., Q149K Q159E G235D A185C) was 10-fold less viable than its counterpart in the C-terminally truncated version *cyCtrC4* and only 5-fold more viable than the clone (pRM4-C) expressing *cyt1Aa* (24).

TABLE 3. Viabilities on IPTG-containing LB plates

Clone[a]	% Survival (mean ± SD)[b]
pUH-*cyCtrC*	86 ± 9
pUH-*cyCtrC12*	29 ± 6
pUH-*cyCtrC3*	17 ± 2
pUH-*cyCtrC4*	6.3 ± 0.7
pUH-*cyCtrC5*	70 ± 6.3
pUH-*cyCtrC6*	83 ± 11
pUH-*cyCtrNC*	106 ± 22
pUH-*cyCtrNC12*	107 ± 38
pUH-*cyCtrNC6*	100 ± 14
pUH-*cyCtrNC4*	0.61 ± 0.046
pRM4-C	0.13 ± 0.056

[a] Clones were plated on LB plates with or without IPTG.
[b] There were four replicates for each experiment.

Morphologies of inclusion bodies in the clones expressing *cyCtrC*. The nonmutated version formed a large inclusion body situated at the *E. coli* cell pole (Fig. 3A to D), while all four mutated versions generated multiple inclusion bodies spread along the cell envelope (e.g., Fig. 3E to H). On the other hand, expressing *cyCtrNC* (Cyt1Ca truncated at both termini according to the proteinase K activation pattern) and its mutated versions did not bring about the formation of inclusion bodies (Fig. 4).

Cell morphology (Fig. 4). Expressing *cyCtrNC12*, *cyCtrNC6*, and *cyCtrNC4* (Table 1) caused pronounced distortions of cell morphology. Three hours after induction by IPTG, about 30% of the cells became elongated, bent, and bulged (Fig. 4), while expressing the mutated versions of *cyCtrC* caused cell elongation only (Fig. 3). This distortion accompanied inhibition of cell divisions upon expression of *cyCtrNC12* and *cyCtrNC6* (not shown) and viability loss upon expression of *cyCtrNC4* (Table 3).

Bioassay of the mutated proteins. None of the *E. coli* clones expressing *cyt1Ca* or any of its mutated versions displayed toxicity against larvae of *A. aegypti* (not shown) or synergistic activity with a strain expressing *cry4Aa* (Fig. 5).

DISCUSSION

The recently discovered open reading frame in pBt054 (5) encodes a protein, Cyt1Ca, which surprisingly is not toxic to

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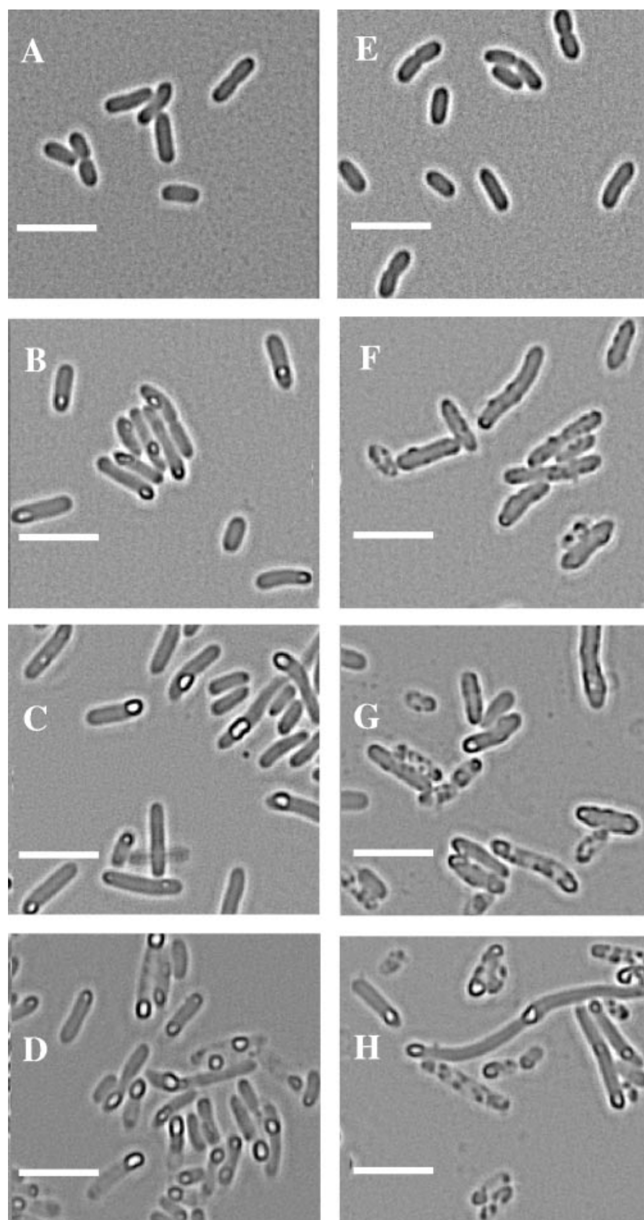

FIG. 3. Morphology of inclusion bodies formed by expressed *cyt1Ca* versions in *E. coli*. Cells harboring pUH-*cyCtrC* (A-D) and pUH-*cyCtrC4* (E-H), uninduced (A, E) and after 2 h (B, F), 4 h (C, G), and 20 h (D, H) of induction by IPTG. Bars, 5 μm.

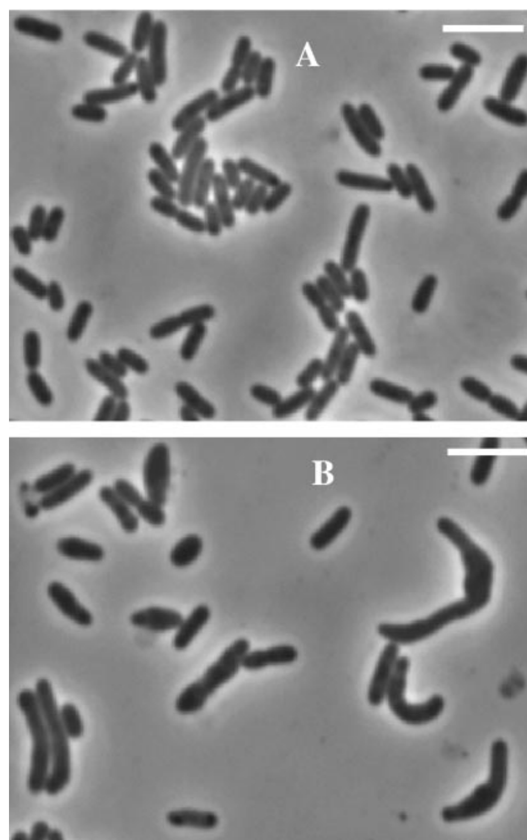

FIG. 4. Cell morphology of *E. coli* cells expressing different versions of *cyt1Ca* 3 h after induction by IPTG: *cyCtrNC* (A) and *cyCtrNC12* (B). Bars, 5 μm.

mosquito larvae or to *E. coli* (23) despite the high homology of its N-terminal domain to the full-length Cyt1Aa (Fig. 1). No Cyt1Ca has yet been identified in *B. thuringiensis* subsp. *israelensis*. The question thus arises of its possible function in the bacterial life cycle, toxicity, or evolution. To start deciphering this enigma, five versions of *cyCtrC* (encoding the N-terminal 244 amino acids that resemble Cyt1Aa) containing combinations of sequential mutations were constructed (Tables 1 and 2; Fig. 1). Expressing them in *E. coli* caused cumulative deleterious effects. Delay or inhibition of cell division in all mutants and loss of viability in some of the mutants (Fig. 2; Table 3) were accompanied by the appearance of multiple inclusion bodies positioned in proximity to the cell envelope (Fig. 3). These results point to a possible interaction of the changed Cyt1Ca with the inner membrane of *E. coli* that may interfere with its division machinery and damage the membrane causing cell lysis as Cyt1Aa does (25).

Initial contact of Cyt1Aa with membranes involves electrostatic interactions of the charged amino acids, situated on its surface, with the lipid head groups (26). The toxin binds in vitro specifically to zwitterionic phospholipids, such as phosphatidylcholine and phosphatidylethanolamine (32). The latter is the major phospholipid in the *E. coli* inner membrane (29) and has been proposed to be localized in segregated domains (36). The interaction of Cyt1Aa with these domains may be responsible for its toxicity against *E. coli* (25). Particular amino acids have been assigned as possible candidates for this interaction (26), among which are K154, E164, and D240 (Fig. 1). Thus, one of the factors causing the absence of Cyt1Ca activity may be the noncharged nature of the amino acids corresponding to the above (Q149, Q159, and G235). Changing them back as in Cyt1Aa indeed partially restored activity of C-terminally truncated Cyt1Ca (Fig. 2 and Table 3).

Not all the chosen substitutions in Cyt1Ca enhance the antibacterial effect. For example, the Q220K substitution in CyCtrC12 (forming CyCtrC6) or CyCtrC4 (CyCtrC5) decreased their killing effects against *E. coli* cells (Table 3). Q220 in Cyt1Ca is homologous to K225 in Cyt1Aa, but the latter is

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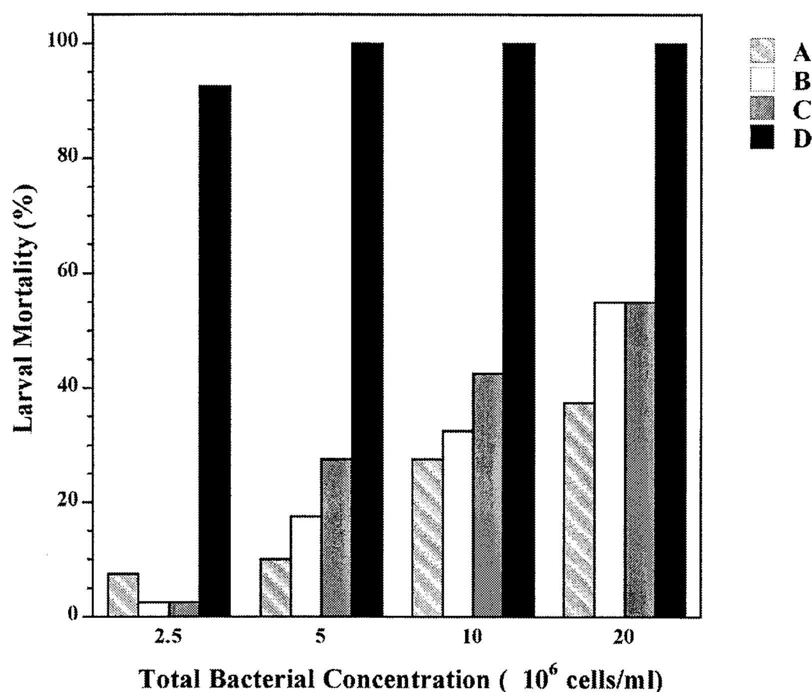

FIG. 5. Mortality of third-instar *Aedes aegypti* larvae upon feeding the larvae with the mixture containing the clone pHE4-A mixed with the following clones: pUHE-24S (A), pUH-*cyCtrC* (B), pUH-*cyCtrC4* (C), and pRM4-C (D). Mixtures were prepared as described in Materials and Methods.

situated in the inner core of Cyt1Aa rather than on its surface (22) (Fig. 1). It therefore seems not to be involved in initial binding to the lipid head groups via electrostatic interactions but in membrane penetration afterwards (22). The effect of the Q220K substitution in Cyt1Ca may thus be more complicated than substitutions of the others. Stronger suppression by Q220K of the quadruple Q149K Q159E G235D A185C than of the double substitution Q149K Q159E in Cyt1Ca may be explained by the lower affinity to the membrane of the amino acid combination C185 K220 than of the combination C185 Q220. This interpretation is supported by the fact that homologous amino acids K225 and C190 (in Cyt1Aa) to Q220 and A185 (in Cyt1Ca), respectively, are contiguous when mapped into the three-dimensional structure of Cyt2Aa, the only Cyt-like protein deciphered (22). However, the analogous suppression of CyCtrC12 by Q220K (Table 3) apparently contradicts this conclusion. It seems as though the original Q220 is more effective against *E. coli* than K220 at a given amino acid context in Cyt1Ca.

Full toxicity of Cyt1Aa in the midgut of the target organism, mosquito larvae, needs a two-end proteolytic activation (1). Similar processing in vitro has been achieved by proteinase K (1), which is why CyCtrNC and its mutant derivatives were constructed (Tables 1 and 2). The deleterious effects of some of these derivatives to *E. coli* were greater than that of the mutated protein truncated just at N244: the division inhibition of *E. coli* expressing these versions of mutated *cyt1Ca* was accompanied by pronounced distortion of cell morphology (Fig. 4) not occurring when any of the mutated versions of *cyCtrC* were expressed. Moreover, viability of cells expressing *cyCtrNC4* was five times lower than those expressing *cyCtrC4* (Table 3). The difference in activities damaging *E. coli* cells between C-terminally truncated and doubly truncated Cyt1Ca can be explained by an aggregation-prone nature of the former compared to the latter (Fig. 3): generation of inclusion bodies may prevent the aggregated protein from efficient interaction with the membrane.

The relative importance of different charged (37), hydrophobic (3) amino acids for Cyt1Aa activity and tryptophans for Cyt2Aa2 activity (30) has been studied systematically by site-directed mutagenesis. Knocking down activities of these proteins by amino acid substitutions revealed rather easily the significance of the replaced amino acids in Cyt function. An attempt was undertaken here to solve the problem the other way around, by restoring the presumably lost function of Cyt1Ca in a learned way. The described deleterious effects of mutated *cyt1Ca* versions on *E. coli* may reflect an evolutionary relationship between Cyt1Aa and Cyt1Ca. Expression of *cyt1Aa* in *E. coli* resulted in abrupt arrest in biomass growth and nucleoid compaction, the mechanism of which probably involves disturbing all or most of the membrane functions (25). These effects were not observed in *E. coli* (not shown) when it expressed even the most bactericidal versions of mutated *cyt1Ca*: *cyCtrC4* and *cyCtrNC4* (Table 3). Expression of these genes exerted lethality on *E. coli* in a much more moderate and slower mode probably because it affects a limited number of membrane functions, such as division machinery and shape determination. Moreover, the affinities of the mutated versions of Cyt1Ca to the bacterial membrane that resulted in the above effects is not adequate to its larvicidity against *A. aegypti* (Fig. 5). The original question posed, what does the so-called *cyt1Ca* open reading frame do in pBtoxis, is thus not resolved yet, and

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further genetic and biochemical analyses are required. However, the approach to acquire bactericidal abilities to totally inactivate Cyt1Ca is very promising in clarifying the yet enigmatic general bactericidal effect of Cyt1Aa.

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