APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2010, p. 3409–3411 Vol. 76, No. 10
0099-2240/10/$12.00 doi:10.1128/AEM.03068-09

Expression in *Escherichia coli* of the Native *cyt1Aa* from *Bacillus thuringiensis* subsp. *israelensis*▽

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The gene *cyt1Aa* is one of the genes in the complex determining the mosquito larvicidity of *Bacillus thuringiensis* subsp. *israelensis*. Previous cloning in *Escherichia coli* resulted in a 48-bp addition upstream, encoding a chimera. Here, *cyt1Aa* was recloned without the artifact, and its toxicity against *Aedes aegypti* larvae and host *E. coli* cells was retested.

Bacillus thuringiensis subsp. *israelensis* is an effective control agent against mosquito and black fly larvae (13). Its larvicidal activity is determined by four major polypeptides of the parasporal crystalline body produced during sporulation: Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa. The encoding genes are mapped on the 128-kb plasmid pBtoxis (3). Cyt1Aa has low toxicity and specificity but is the most synergistic to any of the other three (9) and hence has a crucial role in suppressing resistance to Cry toxins in the targets (19). In addition, Cyt1Aa exerts, a general cytolytic effect on a variety of prokaryotic (5, 20) and eukaryotic (16) cells by a detergent-like mode of action (12). This property makes it potentially useful for treating bacterial infections and even cancerous growths if specificity to tumor cells is raised by genetically fusing Cyt1Aa to targeting ligands (6).

cyt1Aa was cloned in *Escherichia coli*, for the first time, as part of a 10-kb HindIII digestion fragment of pBtoxis on pUC12 (18) and its sequence (GenBank accession number X03182) was determined (17). Attempts to subclone it separately under its own promoter had failed due to the antibacterial activity of Cyt1Aa (1, 8), unless coproduced with a 20-kDa protein encoded by *p20* that is carried on the same HindIII fragment (1, 10). Cloning *cyt1Aa* alone had been achieved only under more-stringent repression conditions, by placing it under the promoter recognized by phage T7 RNA polymerase (8), encoded by the gene that was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) from the chromosome of *E. coli* strain BL21 (15). However, this expression system as such inhibits cell metabolism; hence, investigating the *in vivo* effect of Cyt1Aa on *E. coli* requires a lenient system. The current procedure to engineer restriction sites into PCR primers enables one to easily clone *cyt1Aa* under any desired promoter. To achieve this purpose, we exploited vector pUHE-24S (kindly provided by Stefan Leu), a modified pUHE-24 (University of Heidelberg, Germany) from which one NcoI site had been removed, leaving a unique NcoI site in the translation start codon. pUHE-24 is a descendant of pDS (7) containing a T5 ribosome-binding site (4), two tandem $lacO_1$, and the early T7 promoter P_{A1} (utilizing the usual *E. coli* RNA polymerase) (Fig. 1A).

The HindIII site in the vector multicloning site (MCS), downstream of the desired NcoI site, was used to clone *cyt1Aa* because its reading frame contains an NcoI restriction site 46 nucleotides from the translation start codon ATG. The corresponding HindIII site in the *cyt1Aa* insert was PCR generated in the upstream region of the gene (Fig. 1B). The created clone expressed *cyt1Aa* upon IPTG induction and was successfully used for the subsequent research (10, 11).

However, it was recently determined that this upstream region of *cyt1Aa* along with the rest of the MCS of pUHE-24S was included in-frame with *cyt1Aa* and the vector ATG, extending the gene by 48 nucleotides at its 5′ end, and was thus translated into a Cyt1Aa version with 16 additional amino acids at its N terminus (Fig. 1B). Since this N-terminal tail may adversely affect stability/activity of the protein, it was decided to reclone the native gene without that artifact.

To achieve this end, the *cyt1Aa* intrinsic NcoI site was eliminated by Stratagene's QuikChange site-directed mutagenesis kit. Plasmid pRM4-C (10) was used as a template to produce two adjacent point mutations, eliminating the NcoI restriction site (shown in boldface type) (Fig. 1C) and creating an EcoRV restriction site (underlined), together with the following primers: 5′-GTCCATTAGAA<u>GATATC</u>AAGGTAAAT**CCCTGG**AAAACCCCTC-3′ and 5′-GAGGGGTTTT**CCAGGG**ATTTACCTT<u>GATATC</u>TTCTAATGGAC-3′.

Replacement of nucleotides for the above mutations does not change the corresponding amino acids (compare Fig. 1B and C). The mutations in the generated plasmid [pRM4-C(Mut)] were confirmed by digestion with the restriction endonuclease and by DNA sequence analysis.

cyt1Aa from pRM4-C(Mut) was amplified with Vent polymerase using the following primer pair: 5′-GTTTATCCATGGAAAATTTAAATCATTGTCC-3′ and 5′-CGCGAGGCAGCTCTAGATTAACGC-3′. The PCR products were digested with NcoI and XbaI and inserted into the respective sites of pUHE-24S, generating pRM4-C(Nat).

Transgenic *E. coli*, grown at 37°C in ampicillin-containing LB medium, was induced by IPTG (0.5 mM) at an optical density (at 660 nm) of 0.1 to 0.2 (about 2×10^7 cells ml^{-1}).

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▽ Published ahead of print on 26 March 2010.

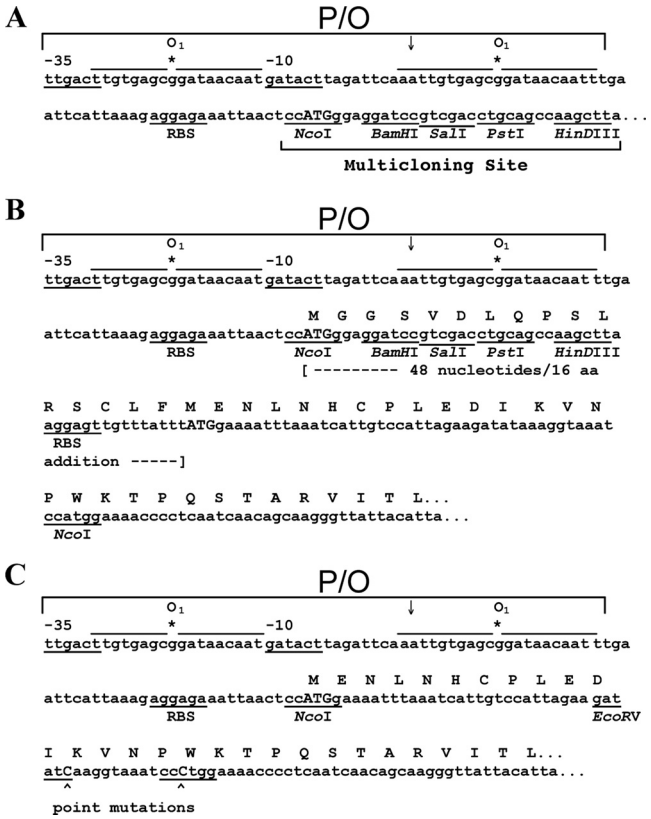

FIG. 1. Essential features of the nucleotide sequences of the promoter/operator/polylinker region of pUHE-24S (A), pRM4-C (B) and pRM4-C(Nat) (C). P/O, the promoter region with −35 and −10 elements and operator regions (O_1) with transcription start (arrow); RBS, T5 ribosome binding site of the vector and the Shine-Dalgarno-like sequence preceding the *cyt1Aa* original translation start codon ATG, shown in uppercase. Asterisks designate the centers of symmetry of $lacO_1$ operators.

Cells were harvested by centrifugation before induction and, after 1, 2, and 4 h, were resuspended in water, and aliquots were boiled (10 min) in a sample treatment buffer. Proteins were separated by SDS-PAGE on 13% gel, electrotransferred onto nitrocellulose membranes, and exposed to antiserum directed against Cyt1Aa (kindly provided by Sarjeet Gill, University of California, Riverside). Alkaline phosphatase-conjugated protein A was used as a detector, and the antigen

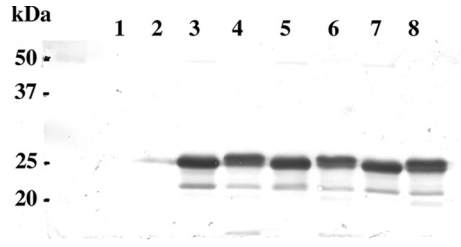

FIG. 2. Immunoblot of Cyt1Aa in clones pRM4-C (even-numbered lanes) and pRM4-C(Nat) (odd-numbered lanes) either uninduced (lanes 1 and 2) or IPTG induced for 1 h (lanes 3 and 4), 2 h (lanes 5 and 6), and 4 h (lanes 7 and 8).

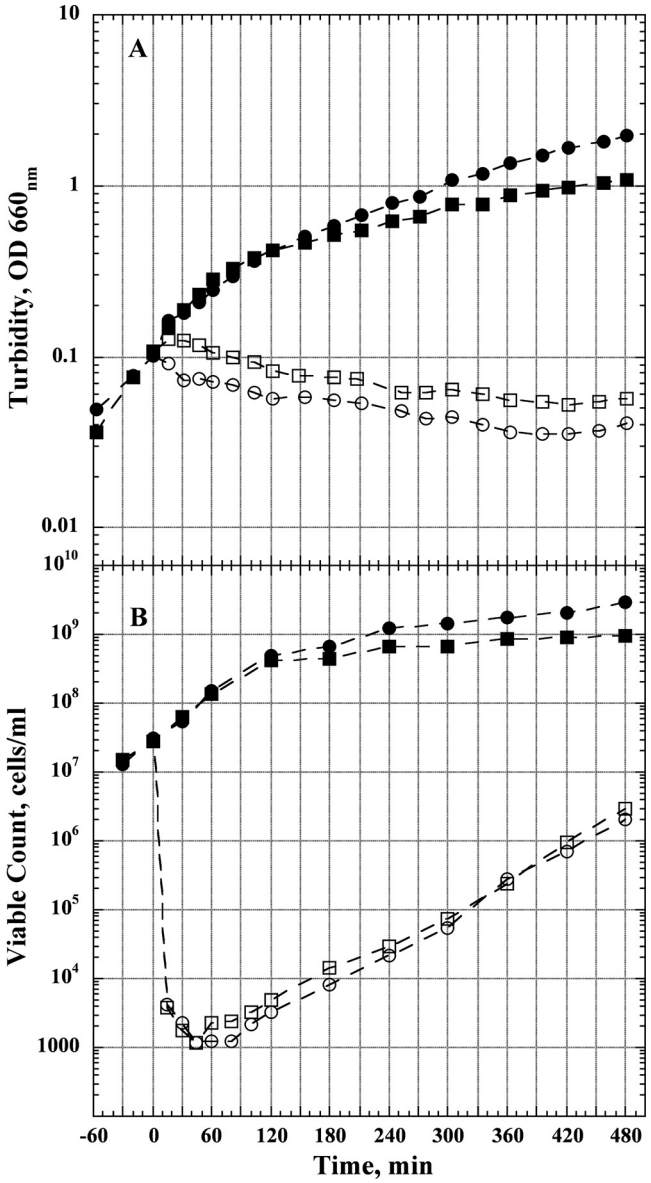

FIG. 3. Mass growth (A) and viable counts (B) of *E. coli* clones, induced (open symbols) with IPTG at time zero and uninduced (closed symbols) harboring either pRM4-C (squares) or pRM4-C(Nat) (circles).

was visualized with chromogenic substrate for alkaline phosphatase.

The immunoblot (Fig. 2) clearly demonstrates one band of ca. 25 kDa produced from pRM4-C(Nat), while the corresponding band representing Cyt1Aa produced from pRM4-C is slightly larger. This is consistent with the suggestion that the initial cloning of *cyt1Aa* created an extended product upon expression. This conclusion was validated by sequencing the respective genes.

The clones pRM4-C and pRM4-C(Nat) were compared regarding their toxicity to *E. coli* (Fig. 3) and synergy to the *cry4Aa*-expressing clone against *Aedes aegypti* larvae (Fig. 4). For these purposes, IPTG-induced clones were tested, respectively, for colony-forming ability (after 24 h of incubation at

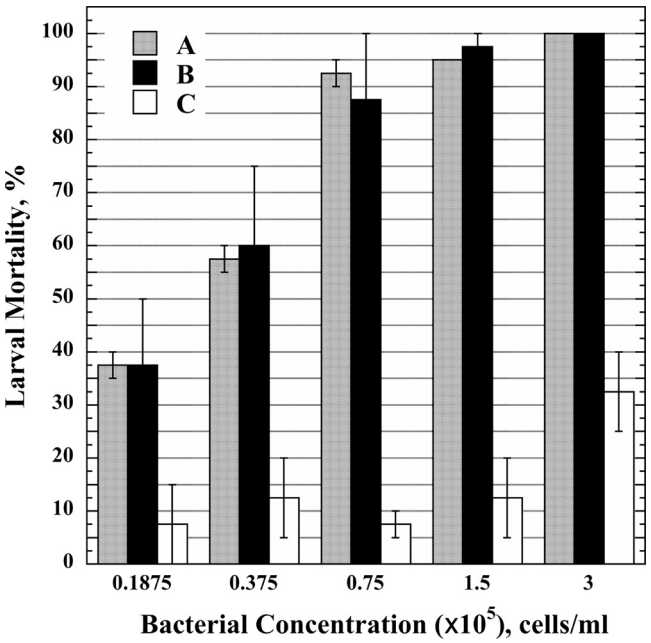

FIG. 4. Mortality of third-instar *A. aegypti* larvae upon feeding them with a mixture containing the clone producing Cry4Aa and one of the following clones: pRM4-C(Nat) (A), pRM4-C (B), and pUHE-24S (empty vector) (C) at a ratio of 1:4 (by cell number). Samples were added to 20 early-third-instar *A. aegypti* larvae in disposable cups with 100 ml sterile tap water, and larval mortality was determined after 24 h at 28°C. Standard error values (bars) were calculated from duplicates for each concentration in a single experiment.

37°C) on LB plates with 100 μg ampicillin ml^{-1} and for killing early-third-instar *A. aegypti* larvae 24 h (at 28°C) after being mixed with the *cry4Aa*-expressing clone in disposable cups with 100 ml sterile tap water. No significant difference was found between the clones, concluding that the additional 16 amino acids do not significantly affect Cyt1Aa activities. Viable counts of induced cells appear to recover (Fig. 3B), most likely due to spontaneous mutants that gained resistance to the lethal effect of Cyt1Aa (11).

Translation of noncoding DNA sequences in-frame with cloned genes can potentially present an obstacle for the study of the corresponding protein, and special attention must be paid to the cloning procedure so that the desired gene product is obtained. The specific modification described here did not change the activity of Cyt1Aa produced in *E. coli*. The unaffected larvicidity against *A. aegypti* is simply explainable since Cyt1Aa undergoes activation by proteolysis in the larval gut; a fragment of about 30 amino acids is cleaved off the N terminus of native Cyt1Aa during processing (2), obviously including the 16 amino acids previously added inadvertently. On the other hand, the unaffected bactericidal level against its host *E. coli* implies that the above-described modification is probably too small to impair proper folding for antibacterial activity of Cyt1Aa. This fact can be used to engineer genetically modified versions of Cyt1Aa fused to short ligand-peptides that target its cytolytic activity to specific cell types as desired (6).

Thanks are due to Monica Einav for providing technical assistance.

This investigation was partially supported by grant no. 2007-037 of the United States-Israel Binational Science Foundation, Jerusalem, Israel.

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