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Contributions of 5'-UTR and 3'-UTR *cis* elements to Cyt1Aa synthesis in *Bacillus thuringiensis* subsp. *israelensis*

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Abstract

The biopesticide used most effectively to control mosquito and blackfly vectors of human diseases worldwide is *Bacillus thuringiensis* subsp. *israelensis*. The high efficacy of this bacterium is due to synergistic interactions among four protein entomotoxins assembled individually into a single parasporal body (PB) during sporulation. Cyt1Aa, the primary synergist, is the most abundant toxin, comprising approximately 55% of the PB's mass. The other proteins are Cry11Aa at ~35%, and Cry4Aa and Cry4Ba, which together account for the remaining ~10%. The molecular genetic basis for the comparatively large amount of Cyt1Aa synthesized is unknown. Here, in addition to the known strong BtI (σ^E) and BtII (σ^K) promoters, we demonstrate a third promoter (BtIII) that has high identity to the σ^E promoter of *Bacillus subtilis*, contributes to the large amount of Cyt1Aa synthesized. We also show that a *cyt1Aa*-BtIII construct was not functional in a σ^E-deficient strain of *B. subtilis*. Comparison of transcription levels and protein profiles for recombinant strains containing different combinations of BtI, BtII and BtIII, or each promoter alone, showed that BtIII is active throughout sporulation. We further demonstrate that a stable stem-loop in the 3'-untranslated region (3'-UTR, predicted $\Delta G = -27.6$) contributes to the high level of Cyt1Aa synthesized.

Graphical Abstract

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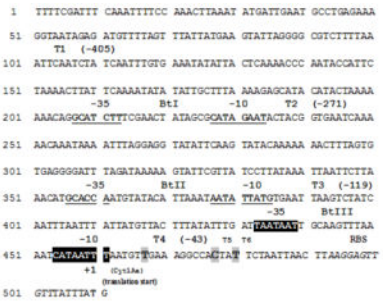
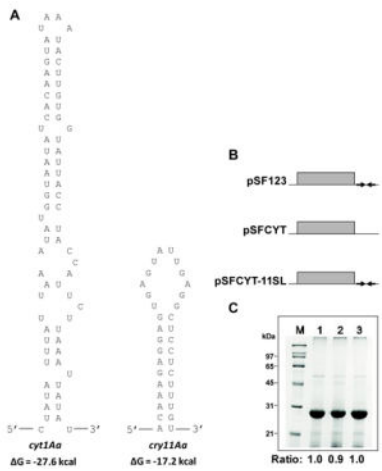
Conflict of Interests

The authors have declared that no competing financial interests exist.



Nucleotide sequence of the 5'-UTR of the *cyt1Aa* gene.



Effect of the 3'-untranslated region (3'-UTR) of the *cyt1Aa* gene on synthesis of Cyt1Aa in *B. thuringiensis*

Keywords

Bacillus thuringiensis subsp. *israelensis*; promoters; transcriptional terminator; 5'-UTR; 3'-UTR; *cyt1Aa*

1. Introduction

The bacterium *Bacillus thuringiensis* subsp. *israelensis* produces a parasporal body (PB) highly toxic to larvae of nematocerous dipterans (Goldberg and Margalit, 1977; Schnepf et al., 1998). Its lethality to mosquitoes, blackflies and midges is due to synergistic interactions among four major PB proteins, three Cry toxins (Cry11Aa, Cry4Aa, Cry4Ba) and the lipophilic Cyt1Aa cytolytic toxin, the primary synergist (Wu and Chang, 1985; Ibarra and Federici, 1986; Crickmore et al., 1995). Cyt1Aa constitutes ~55% of the PB, whereas Cry11Aa accounts for ~35%, and Cry4Aa and Cry4Ba together account for the remaining ~10% (Federici et al., 1990; Wu and Federici, 1993; Dervyn et al., 1995; Diaz-Mendoza et al., 2012).

The molecular genetic basis for the comparatively large quantity of Cyt1Aa in the PB is unknown. Previous studies have identified several factors that influence Cyt1Aa synthesis, including the level of *cyt1Aa* expression, transcript stability, and co-synthesis of a 20-kDa helper protein, often referred to as a "chaperone-like" protein that prevents cytolysis of the host bacterial cell during Cyt1Aa synthesis (Adams et al., 1989; Wu and Federici, 1993; Baum and Malvar, 1995). Most studies of endotoxin synthesis in *B. thuringiensis* have

focused on *cry* genes, for which transcription is typically controlled by two strong sporulation-dependent promoters (BtI and BtII) activated by σ factors that share high homology with, respectively, the σ^E and σ^K of *Bacillus subtilis* (Wong et al., 1983; Brown and Whiteley, 1988; 1990; Adams et al., 1991; Brown, 1993; Helmann and Moran, Jr., 2002). With respect to mRNA stability, a sequence referred to as STAB-SD was identified in the 5'-UTR of *cry3A* that enhances Cry3A synthesis (Agaisse and Lerelcus, 1996; Park et al., 1998). In addition, 3'-UTRs of most *cry* genes contain inverted repeats that form secondary structures (Agaisse and Lereclus, 1995) that stabilize corresponding mRNAs while also functioning as transcription terminators (Wong and Chang, 1986; Park et al., 2000). *Trans* factors that act during or after translation include the 20-kDa and 29-kDa helper proteins encoded by, respectively, the *cry11Aa* and *cry2Aa* operons. The 20-kDa protein enhances Cyt1Aa and Cry11Aa synthesis (McLean and Whiteley, 1987; Adams et al., 1989; Visick and Whiteley, 1991; Wu and Federici, 1993), whereas the 29-kDa protein apparently serves as a scaffold for Cry2Aa crystallization (Crickmore and Ellar, 1992; Ge et al., 1998). Finally, as yields of different Cry proteins synthesized with the same expression system vary markedly, biochemical characteristics of the protein itself, such as its primary structure and sensitivity to proteolytic degradation, affect yield and thus the amount of crystal toxins obtained per cell (Delécluse et al., 1993; Agaisse and Lereclus, 1995; Baum and Malvar, 1995; Park et al., 1999).

The accumulation of high levels of Cyt1Aa suggests that it may be less susceptible than Cry proteins to proteolysis. Moreover, it is also likely that *cis* and *trans* elements facilitate stability of its transcript and nascent protein. The BtI and BtII promoters and 20-kDa protein are, however, insufficient to explain the differentially large amount of Cyt1Aa relative to Cry4Aa, Cry4Ba and Cry11Aa in *B. thuringiensis* subsp. *israelensis* because these proteins are all encoded by genes regulated by these two promoter types, and synthesized in the presence of the 20-kDa protein (Yoshisue et al., 1993a; 1993b; Devyn et al., 1995; Wu and Federici, 1995; Poncet et al., 1997; Yoshisue et al., 1997). To determine whether other *cis*-acting elements affect Cyt1Aa synthesis and crystallization and might be genetically manipulated to lower this protein's yield to improve PB toxicity, we constructed several recombinant *cyt1Aa* clones containing different combinations of its 5-UTRs and 3'-UTR sequences. In the present study we (i) identified a third functional σ^E promoter (BtIII), (ii) analyzed the effects of BtI, BtII, BtIII, and combinations of these, and (iii) evaluated the effect of the strong 3'-UTR stem loop on net synthesis of Cyt1Aa. Our data demonstrate specific genetic modifications of *cyt1Aa* can be used to control the amount of Cyt1Aa synthesized and size of the crystal it forms.

2. Materials and Methods

2.1. Bacterial strains and culture media

The DH5α strain of *Escherichia coli* (Bethesda Research Laboratories, Grand Island, NY) was used for cloning and amplifying plasmid DNA. The strains of *B. thuringiensis* and *B. subtilis* used in this study were obtained from the *Bacillus* Genetic Stock Center (Ohio State University, Columbus, OH). The acrystalliferous 4Q7 strain of *B. thuringiensis* subsp. *israelensis* was used for producing Cyt1Aa. Plasmid DNAs extracted from the crystalliferous

4Q5 strain of *B. thuringiensis* subsp. *israelensis* and the HD-73 strain of *B. thuringiensis* subsp. *kurstaki* were used, respectively, as templates to obtain *cyt1Aa* and the 20-kDa protein genes, and *cry1Ac* promoters. *B. subtilis* strains included σ^E (IS60; *leuB8 spoIIG41 tal-1*), σ^K (IS38; *spoIIIC94 trpC2*), and σ^F (IS86; *sigF1 trpC2*) mutants. The *B. subtilis* σ^G mutant strain (*spoIIIGΔ1 trpC2*) was a gift from Dr. Peter Setlow (Department of Molecular, Microbial, and Structural Biology, University of Connecticut Health Center, Farmington, CT). All strains were maintained on nutrient agar (Becton Dickinson, Sparks, MD) throughout the study. LB medium (Becton Dickinson, Sparks, MD) was used for growing *E. coli* and extracting plasmid DNA. For transformation of *B. thuringiensis* and *B. subtilis*, brain heart infusion medium (BHI; Becton Dickinson, Sparks, MD) was used to recover cells. The nutrient broth/glucose (NBG) medium (Park et al., 1999) was used for Cyt1Aa synthesis in *B. thuringiensis*. Erythromycin (25 μg/ml) and ampicillin (100 μg/ml) were used for selection when required.

2.2. PCR and plasmid construction

PCR primers used in this study are shown in Table 1, and PCR was performed using Vent (exo^-) DNA polymerase (New England Biolabs, Beverly, MA). The thermal program was one cycle at 94 C for 5 min, followed by 30 cycles at 94 C for 1 min, 55 C for 1 min, 72 C for 3 min, and a final extension step at 72 C for 7 min. Amplicons were phosphorylated with T4 polynucleotide kinase (Biolabs) to facilitate ligation. The *cyt1Aa* gene, the *cry1Ac* promoters and the 20-kDa protein gene were amplified using plasmid DNAs of, respectively, the HD-73 and 4Q5 strains. Two separate amplicons were generated. One was the *cry1Ac* promoter region, amplified with primers 5'-CRY1Ac-P-F and 3'-CRY1Ac-P-R, which contains a *Sal*I site at the 5' end (143 bp). The other was the 20-kDa protein gene open reading frame (ORF) and its downstream transcription termination sequences that contain a *Pst*I site at the 3' end (1,247 bp), amplified with primers 5'-20kDa-F and 3'-20kDa-R. Both were inserted simultaneously into pUC19 (New England Biolabs, Beverly, MA). The fragment containing the *cry1Ac* promoter driving expression of 20-kDa protein gene (*Pcry1Ac-20kda*) was digested with *Sal*I and *Pst*I, and cloned into the same sites in pHT3101 (Lereclus et al., 1989).

To obtain the *cyt1Aa* gene containing different combinations of its BtI, BtII and BtIII promoters, four forward primers were used with the same reverse primer (CYT1-R). The four *cyt1Aa* amplicons, each containing a different length of the upstream promoter region [1,408 bp (primer CYT1-1-F), 1,144 bp (primer CYT1-2-F), 1,013 bp (primer CYT1-3-F) and 941 bp (primer CYT1-4-F)] were digested with *Xba*I and *Sal*I and introduced into the same sites in pHT3101 containing *Pcry1Ac-20kda*. The resulting plasmids containing these constructs were named, respectively, pSF123 (9,556 bp), pSF23 (9,292 bp), pSF3 (9,161 bp), and pSF (9,089 bp), harboring, respectively, BtI, BtII and BtIII, BtII and BtIII, BtIII, and no promoters (Fig. 1 and 2). Plasmids with only BtI (pSF1, 9375 bp) or BtII promoter (pSF2, 9240 bp) or BtI+II (pSF12, 9509 bp) were constructed using the following two steps: (i) three separate fragments containing the BtI (286 bp), or BtII (151 bp), or BtI+II (420 bp) promoter region were amplified by PCR with CYT1-sigE-F and CYT1-sigE-R, or CYT1-sigK-F and CYT1-sigK-R, or CYT1-sigE-F and CYT1-sigK-R, respectively, and (ii) these 3 amplicons were digested with *Sac*I and *Xba*I and cloned separately into the same sites in

pSF which included the *cyt1Aa* ORF without the promoter region, and *Pcry1Ac-20kda* (Figs. 1 and 2).

In addition, constructs were made to determine the effects of either the absence of the *cyt1Aa* 3'-UTR stem loop or the presence of the weaker *cry11Aa* 3'-UTR stem loop (see Fig. 8) on Cyt1Aa synthesis. The plasmid without the *cyt1Aa* stem-loop (pSFCYT, 9,406 bp) was constructed by the following two steps: (i) a *cyt1Aa* fragment without its own stem-loop structure was amplified with CYT1-1-F and Cyt-nostem-R primers, and (ii) the amplicon was digested with *Xba*I and *Sal*I and ligated to the same sites in pHT3101, which contained the *Pcry1Ac-20kda*. The plasmid containing the *cyt1Aa* ORF with the *cry11Aa* stem loop at the 3' end (pSFCYT-11SL, 9,606 bp) was obtained as follows: (i) a fragment containing a *cry11Aa* stem-loop structure (200 bp) was amplified with 11Astem-F and 11Astem-R using pWF53 (Wu and Federici, 1995) as the template, (ii) the amplicon was digested with *Xba*I and *Sal*I and ligated to the same sites in pUC19 which contained *Pcry1Ac-20kda*, (iii) the *cry11Aa* stem loop/*Pcry1Ac-20kda* construct was digested with *Xba*I and *Pst*I and ligated to the same sites in pHT3101, (iv) a fragment of *cyt1Aa* ORF that lacks its own stem loop structure at the 3' end was amplified with CYT1-sigE-F and CYT-11Astem-R, and (v) the amplicon was digested with *Sac*I and *Xba*I and ligated to the same sites in pHT3101 which included the *cry11Aa* stem loop and *Pcry1Ac-20kda*. The integrity of each construct was confirmed by restriction and nucleotide sequence analyses.

2.3. RNA analyses

Each *B. thuringiensis* strain with the different number of promoters was grown in 4 ml of BHI medium overnight and 100 μl of the pre-culture was used to inoculate 50 ml NBG broth. Ten ml from each culture was harvested at 16, 19, 22, 25, 28, 31, 34, and 37 h post inoculation (p.i.). These sampling time points were chosen based on a preliminary experiment in which cell development and sporulation were monitored using phase contrast microscopy, and Cyt1Aa synthesis assessed by SDS-PAGE analysis. At 28 h p.i., bipyramidal Cyt1Aa crystals and spores were observed in all strains with promoters, and spores only in the control strain lacking *cyt1Aa* promoters (4Q7/pSF). To extract total RNA, cells were centrifuged at 5,500 g at 4°C for 15 min. Lysozyme (10 mg/ml) was added to cell pellets and the content was mixed thoroughly and incubated at 37°C for 1 h. The mixture was suspended in 1 ml of TRIzol® reagent (Invitrogen, Carlsbad, CA) and 600 μl aliquots were transferred to 2 ml microcentrifuge tubes. The solution was mixed thoroughly and incubated at room temperature for 5 min. Then, 200 μl of chloroform was added, mixed thoroughly, incubated at room temperature for 3 min, and centrifuged at 15,000 g at 4°C for 15 min, after which the aqueous phase was transferred to fresh microcentrifuge tubes. The addition of TRIzol® and chloroform, followed by the centrifugation described above, was repeated once. The aqueous phase was transferred to fresh tubes and an equal volume of isopropanol was added, then the samples were centrifuged at 16,000 g at 4°C for 10 min. Precipitates were washed with 75% ethanol, centrifuged at 6,300 g at 4°C for 5 min, dried, and dissolved in 100 μl of DEPC-treated double-distilled water. RNA concentrations were determined by absorbance at 260/280 nm with a spectrophotometer (DU® 530, Beckman Coulter, Fullerton, CA). RNA analyses were performed as described previously (Park et al., 1998) with minor modifications, i.e., 5 μg of each RNA sample was blotted and antisense-

cyt1Aa DNA probe was used, and intensities were quantified using the AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA). For statistical analyses, at least three separate replicate experiments were performed, each on different days, using different cultures.

2.4. BtIII transcripts

The 5' transcription initiation site of the mRNA synthesized from the BtIII promoter was determined using the protocol described by Zhang and Chiang (1996). Briefly, DNAse I-treated RNAs were reversed transcribed using reverse primer MDMcyt1R (5'-CAAAATCTGTAGAAGTGGGAACTAATGC-3'; complementary positions 220 to 193 downstream from the translational start site of the *cyt1Aa* open reading frame) and the ProtoScript kit (Biolabs) according to manufacturer's protocol. RNA was removed by treatment with RNAse H (Invitrogen), and single-stranded (ss) cDNA was fractionated in a 2% agarose gel and purified using the QIAquick Gel Extraction kit (Qiagen). Oligo B (phosphorylated 5'AGGGTGCCAACCTCTTCAAG-3') was ligated to the 5' end of ss cDNA using T4 RNA ligase (New England Biolab) according to manufacturer's protocol. PCR was performed with Taq DNA polymerase (Promega) and the ss cDNA and RNA preparation (negative control) using a nested primer (DBGEcytIIR, 5'-CTTGCTGTTGATTGAGGG-3'; complementary positions 74 to 56 downstream from the translational start site of the *cyt1Aa* open reading frame, and Primer C (5'-CTTGAAGAGGTTGGCACCCT-3', complement of Oligo B). The PCR product was separated in 2% agarose, purified with the Gel Extraction kit, and cloned in pGEM-T Easy (Promega) for nucleotide sequencing at the IIGB Instrumentation Facilities, Institute for Integrative Genome Biology, University of California, Riverside. Several clones were sequenced to determine the transcription initiation site.

2.5. Site-directed mutagenesis of BtIII promoter

Base substitutions in the putative -35 and -10 sequences were performed using primer pairs DBBT3M and CYT1-R and DBBT3TB and CYT1-R, and the wild-type sequence with primers DBBT3 and CYT1-R (Table 1, Fig. 6) and Phire HotStart II polymerase (Thermo Scientific). The amplicons were digested with *Xba*I and *Sal*I and cloned in the same sites in pSF3. The integrity of constructs was confirmed by nucleotide sequence analysis. The 4Q7 strain was electroporated with the resulting plasmids and tranformants 4Q7/SF3μ35 (mutated BtIII -35 box), 4Q7/SF3μ10 (mutated BtIII -10 box) and 4Q7/SF3a (wild-type BtIII -35 and -10 boxes) (Fig. 6) were selected as described above.

2.6. Scanning electron microscopy and quantification of protein synthesis by B. thuringiensis

After obtaining synchronous populations by pre-culturing in BHI medium, each strain was grown in 50 ml of NBG medium at 30 C for 5 days to obtain spores and crystals from lysed cells. For scanning electron microscopy, Cyt1Aa crystals were purified as previously described (Park and Federici, 2000), and micrographs were taken with a Philips XL30-FEG scanning electron microscope to determine crystal shape and size. Of a total of 45 to 105 crystal images per construct, 20 whose axes were exactly or nearly at 90 degree to the field of the view were selected for measurement for each strain.

For protein gels, one ml or 500 μl of lysed culture was centrifuged at 10,000 g for 5 min. The pellet was re-suspended in 60 μl of 5x sample buffer (Laemmli, 1970) and boiled for 5 min, then centrifuged at 10,000 g for 5 min. Ten μl of each supernatant was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% gel and bands were visualized by Coomassie blue staining. To normalize the amount of Cyt1Aa produced by each strain, 12% SDS-PAGE was performed using series of diluted bovine serum albumin (BSA; New England Biolabs, Beverly, MA) with known amounts and scanned. The standard curve was created using the values obtained from the known BSA concentrations to estimate Cyt1Aa yield. All protein gels from five separate experiments were analyzed with an AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA) as described previously (Park et al., 2000), and representative results are shown here.

2.7. Spore counts

Viable spore counts were determined as previously described (Park et al., 1998). Briefly, cells were grown in 50 ml of NBG in 250-ml flasks shaken at 250 rpm for 5 days at 30°C. A 1-ml volume of the culture was then placed in a 1.5-ml tube, heated at 60°C for 20 min, diluted, and plated on nutrient agar. Colonies were counted after 12 h of growth at 30°C. Three replicates were performed and data were analyzed with the Super ANOVA program (Abacus Concepts, Berkeley, Calif.).

2.8. Statistical analyses

Statistical analyses were performed to determine Cyt1Aa's crystal size, spore count, Cyt1Aa and Cry11Aa yields on SDS-PAGE, and RNA dot blot by using Duncan new multiple range and Tukey-Kramer methods with the Super ANOVA program (Abacus Concepts, Berkeley, CA).

2.9. Cyt1Aa synthesis in σ factor mutant strains of B. subtilis

The four asporogenic σ factor-deficient mutant strains of *B. subtilis* were transformed with pSF3, which contained the BtIII promoter to drive expression of *cyt1Aa* and *Pcry1Ac-20kda*. Transformants were grown in 25 ml of GYS medium [0.1% glucose, 0.2% yeast extract, 0.05% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.002% $MgSO_4$, 0.005% $MnSO_4$, 0.008% $CaCl_2$] at 30 C for 3 days. Yields of Cyt1Aa were examined as described above for *B. thuringiensis*, except that 4 to 6 ml of the cultures was subjected to 12% SDS-PAGE analysis. Western blot was performed to detect Cyt1Aa using rabbit anti-Cyt1Aa antibody, as described previously (Park et al., 2000).

3. Results

3.1. BtIII is a third functional promoter for cyt1Aa

To identify potential promoters that *B. thuringiensis* σ factors might recognize, the nucleotide sequence of the 5'-UTR of *cyt1Aa* was examined, focusing on sequences immediately upstream from the four different transcripts reported previously (Waalwijk et al., 1985; Ward and Ellar, 1986; Ward et al., 1986; Dervyn et al., 1995). No easily recognizable promoter sequences were found immediately upstream from the transcriptional start site [T1] identified by Ward and Ellar (1986) in *E. coli* (Fig. 1A). However, the -35 and

-10 sequences conserved among known BtI and BtII promoters were found immediately upstream from the transcripts mapped at, respectively, -271 bp (T2), as identified in *B. thuringiensis* and *E. coli* (Ward and Ellar, 1986; Dervyn et al., 1995), and -119 bp (T3), as identified in *B. thuringiensis* and *B. subtilis* (Ward and Ellar, 1986; Dervyn et al., 1995), from the translational start site (Fig. 1A). When the nucleotide sequence between the transcripts starting at -119 bp (T3) and -43 bp (T4) (Waalwijk et al., 1985; Ward et al., 1986) was examined (Fig. 2A, B), a possible third promoter (BtIII) sequence was identified. The putative BtIII -35 (TAATAAT)and -10 (CATAATTT) boxes, separated by 14 bases, showed high identity of, respectively, 100% and 75%, with the consensus sequence [(T/A)(A/C)ATA(A/T)(A/T)–14 bp–(CATACA(A/C)T] of promoters recognized by *B. subtilis* σ^E (Haldenwang, 1995; Helmann and Moran, Jr., 2002; Makita et al., 2004).

To determine whether BtIII was functional, a series of constructs was generated to compare transcription and Cyt1Aa synthesis directed by this promoter alone, either BtI or BtII, and combinations of these, i.e., BtI+II, or BtII+III and BtI+II+III (Figs. 1 and 2). Each of these constructs was electroporated into 4Q7, after which transcription and Cyt1Aa crystal formation were monitored during sporulation of the following recombinants: BtI+II+III (4Q7/pSF123), BtI+II (4Q7/pSF12), BtII+III (4Q7/pSF23), BtI (4Q7/pSF1), BtII (4Q7/pSF2), BtIII (4Q7/pSF3), and a control lacking BtI, BtII and BtIII to drive *cyt1Aa* expression (4Q7/pSF). Analyses of RNAs obtained from three separate experiments of cultures grown on three different days were performed to determine relative *cyt1Aa* transcript levels for these strains (Figs. 2 and 3). The *cyt1Aa* transcript was not detected in preparations from 4Q7/pSF. Promoters in the remaining 6 strains were functional, as *cyt1Aa*-specific transcripts were detected throughout the sporulation phase of growth (16-37 h). Apparent maximal transcript levels were noted at ~31 h with BtI+II+III (4Q7/pSF123), BtI+II (4Q7/pSF12) and BtI (4Q7/pSF1). For BtII+III (4Q7/pSF23) and BtII (4Q7/pSF2), increases in transcript levels were still observed ~37 h, the maximum time surveyed in this study; based on the trend indicated by the graphs it is likely that increases in these two transcripts continued after this time (Fig. 3). For BtIII (4Q7/pSF3), the *cyt1Aa* transcript levels were consistently lower when compared to the other constructs, especially from ~30–37 h, with the exception of BtII (4Q7/pSF2), for which the lowest levels were observed from 20–27 h (Fig. 3). Regardless, the results clearly demonstrated that BtIII contained functional *cis* elements active throughout sporulation.

3.2. Structural characteristics of Cyt1Aa crystals synthesized using different promoter constructs

When grown under the same conditions, each strain harboring a different promoter construct produced Cyt1Aa crystals (Fig. 4). Cyt1Aa crystals were absent in 4Q7/pSF, which lacked the BtI, BtII and BtIII promoters. The shapes and sizes of Cyt1Aa crystals produced by expression of each promoter construct were determined by scanning electron microscopy (Fig. 4). All crystals had the bipyramidal shape characteristic of Cyt1Aa (Wu and Federici, 1993). However, statistically significant differences in size were observed among the crystals produced in different strains (Table 2). These experiments demonstrated that the BtIII promoter alone was capable of driving the synthesis of a significant quantity of Cyt1Aa, as assessed by crystal size, even though the crystals produced using this promoter were smaller

than those produced by the other constructs. Specifically, the length and width of crystals produced in BtIII (4Q7/pSF3) were about 6–31% and 7–33% shorter than, respectively, those produced in BtII (4Q7/pSF2) and BtI (4Q7/pSF1). Interestingly, crystals produced in BtI+II (4Q7/pSF12) were significantly larger than those produced using all three promoters, but they were not significantly different in size compared to those produced using BtI alone. Larger crystals were observed in the strain with the BtI promoter (4Q7/pSF1), compared with those BtII (4Q7/pSF2) or BtIII (4Q7/pSF3) (Table 2).

In addition to measuring the size of crystals produced by each construct, the level of Cyt1Aa synthesis in each strain was assessed using protein profiles obtained by SDS-PAGE in 3 separate experiments (Fig. 5). Overall, a similar trend was observed among these experiments, with values from representative experiments reported here. Cyt1Aa was not produced in the strain lacking a Bt promoter (4Q7/pSF). The BtIII promoter strain (4Q7/pSF3) produced approximately 6–23% lower amounts of Cyt1Aa than that produced by most of the other strains. Interestingly, however, ~12% more Cyt1Aa was produced per unit medium by this strain compared to the BtII strain. Furthermore, as determined by SDS-PAGE, no significant difference in Cyt1Aa synthesis was observed by BtI (4Q7/pSF1), BtII (4Q7/pSF2) and BtIII (4Q7/pSF3) from day 2 to day 5 (data not shown).

To explain the discrepancy between the yield of Cyt1Aa protein and the size of the crystals obtained with the different promoter constructs, the number of spores produced per unit medium by each strain was determined. The strain (4Q7/pSF3) that synthesized the smallest crystals using the BtIII promoter alone produced more viable spores when compared to the other recombinants, for which the relative spore counts were 0.45 (BtI+II+III), 0.21 (BtI+II), 0.47 (BtII+III), 0.37 (BtI) and 0.7 (BtII) (Table 2). In addition, the amount of Cyt1Aa synthesized per spore in each recombinant was quantified (Table 2) using a standard curve based on known concentrations of bovine serum albumin (BSA), SDS-PAGE gel analysis of Cyt1Aa (Fig. 5), and the number of spores produced (Table 2). The collective data showed the yield was significantly lower with BtIII when compared with BtI/BtIII, with a relative ratio of 1:5.2, and interestingly when compared with BtI/BtII/BtIII the ratio was 1:2.9. Furthermore, the yields of Cyt1Aa protein per spore correlated well with crystal size, i.e., the smaller the crystal, the higher the number of spores (Table 2).

3.3 BtIII transcripts

In addition to the transcript T4 (6 bases downstream from the -10 box) previously reported (Waalwijk et al., 1985; Ward et al., 1986), two additional transcripts were identified in BtIII (4Q7/pSF3) (Fig. 1). T5 and T6 mapped at, respectively, 16 and 19 bases downstream from the -10 box. Based on their relative distances from the putative -10 sequence of BtIII, it is possible that T5 and T6 are degradation products of the T4 transcript.

3.4. BtIII -35 and -10 sequences are essential cis elements of a σ^E-specific promoter

Comparison of the -35 and -10 consensus regions of the BtIII promoter with those promoters of *B. subtilis* (Fig. 1) suggested that this third promoter could be activated by σ^E-like factors. As *cyt1Aa*-specific transcripts were detected in *B. subtilis* (Ward et al., 1986), we utilized mutant strains of this species each lacking a known functional σ factor gene. The high

homology and spacing of BtIII's proposed -35 and -10 boxes to corresponding consensus sequences of *B. subtilis* (Fig. 1) strongly suggested these were essential for promoter function. Therefore, multiple site-specific nucleotide base substitutions were introduced in either the -35 or -10 box (Fig. 6A). Strains transformed with the mutant constructs (4Q7/pSF3μ35 and 4Q7/pSF3μ10) lacked visible crystals, and little or no Cyt1A was detected by Western blot analyses (Fig. 6B).

Mutants of *B. subtilis* each deficient in a specific σ factor were transformed with the BtIII promoter construct, pSF3, and Cyt1Aa synthesis of transformants was analyzed by SDS-PAGE and Western blot analysis to determine whether the BtIII was recognized by σ^E, σ^K, σ^F or σ^G factor. The presence of Cyt1Aa was observed in all strains except for the σ^E-deficient strain (Fig. 7).

3.5. Role of cyt1Aa 3'-UTR stem loop on Cyt1Aa synthesis

To determine whether the *cyt1Aa* 3'-UTR stem-loop contributed to high level of Cyt1Aa synthesis, potentially by stabilizing *cyt1Aa* mRNA, the following plasmid vectors were constructed: (i) pSFCYT in which *cyt1Aa* ORF was under the control of its three promoters but lacked its 3' stem loop, and (ii) pSFCYT-11SL in which *cyt1Aa* ORF was under the control of its three promoters, but its native 3' stem loop was substituted with the weaker stem-loop of the *cry11Aa* operon (Fig. 8A, B). The production of Cyt1Aa resulting from these two constructs was compared with that of BtI+II+III strain that contained the native 3' stem-loop (Fig. 8C). Results of triplicate cultures showed Cyt1Aa synthesis per unit medium by 4Q7/pSFCYT (no stem-loop; lane 2) was approximately 10% less than that that produced by 4Q7/pSF123 and 4Q7/pSFCYT-11SL, both of which produced similar amounts of the toxin.

4. Discussion

In this study we demonstrated that *cyt1Aa* contains a previously unknown third functional promoter (BtIII). Earlier studies of the 5'-UTR of *cyt1Aa* suggested expression of this gene was primarily under control of typical tandem BtI and BtII promoters activated by, respectively, σ^E and σ^K transcription factors (Brown and Whiteley, 1988; 1990; Baum and Malvar, 1995; Dervyn et al., 1995). Four different transcripts, with only two attributed to BtI and BtII promoter (Fig. 1A), were identified in heterologous and native genetic backgrounds, respectively, *E. coli* and *B. subtilis* (Ward et al., 1986), and *B. thuringiensis* (Waalwijk et al., 1985; Ward and Ellar, 1986; Dervyn et al., 1995). This suggested there could be additional promoter(s) driving *cyt1Aa* expression in *B. thuringiensis*. Consequently, in addition to the two classical *B. thuringiensis* promoters, our promoter constructs, including those with mutant -35 and -10 boxes, and transcript analysis confirmed the existence of a functional BtIII promoter immediately upstream from the transcriptional start site (T4, Fig. 1A) that was identified previously in *B. thuringiensis* (Waalwijk et al., 1985) and *B. subtilis* (Ward et al., 1986).

Our results also suggest that the presence of three active promoters (BtI, BtII, and BtIII), together with the 20-kDa chaperone-like protein which functions post-translationally (McLean and Whiteley, 1987; Adams et al., 1989; Visick et al., 1991; Wu and Federici,

1995; Wirth et al., 1997), collectively contribute to the high level of Cyt1Aa synthesis and crystallization in *B. thuringiensis* subsp. *israelensis* strain 4Q7 (Bti 4Q7). As larger crystals were observed in Bti 4Q7 transformants harboring constructs that lacked the BtIII promoter, we are unable to assess the specific contribution of BtIII in the overall synthesis of Cyt1Aa. Further studies requiring disruption of the BtIII promoter sequence in *cyt1Aa* in pBtoxis, the toxigenic plasmid in wild-type *B. thuringiensis* subsp. *israelensis* (Berry et al. 2002), are required to conclusively determine its role in Cyt1Aa synthesis.

The three *cyt1Aa* promoters do not overlap, unlike those of *cry4Aa*, *cry4Ba* and *cry11Aa* (Fig. 9, and Wong et al., 1983; Baum and Malvar, 1995; Dervyn et al., 1995). Importantly, each is active during the sporulation phase of growth (Fig. 3), the period during which high levels of Cyt1Aa are synthesized and crystallize. To our knowledge, the combination and order of functional σ^E, σ^K, and "σ^E-like" promoters, respectively, BtI, BtII and BtIII, have not been reported previously, although a putative σ^H-type element was identified in *cry1Ac*, a gene that also contains σ^E and σ^K promoters (Perez-Garcia et al., 2010). Among *cyt* sequences available in databases including *cyt1Aa* studied here, putative σ factor-binding sequences have been identified for at least five. Two recognized by σ^E and σ^K were identified in *cyt1Aa2* and *cyt2Aa1* (Ward et al., 1986; Koni and Ellar, 1993), but only a single promoter recognized by σ^E, which was found in the upstream region of *cyt1Ab1*, *cyt2Ba1*, and *cyt2Bb1* (Cheong and Gill, 1997; Guerchicoff et al., 1997; Thiery et al., 1997). In addition, at least six others (*cyt1Ab1*, *cyt1Ca1*, *cyt2Aa1*, *cyt2Ba1*, *cyt2Bb1* and *cyt2Bc1*) contain putative promoter sequences that could be recognized by σ^E, based on our sequence analysis (data not shown).

The high homology and spacing of BtIII's -35 and -10 boxes to consensus sequences of known *B. subtilis* promoters (Fig. 1B) indicated that BtIII could be activated by σ^E. Indeed, the synthesis of Cyt1Aa in *B. subtilis* mutant strains, 1S38, 1S86, spoIIIGΔ1, deficient in, respectively, σ^K, σ^F and σ^G function, but not in the σ^E–deficient 1S60 strain transformed with pSF3 (Fig. 7), provides strong evidence that transcription from BtIII is regulated by σ^E. Although it is possible that BtIII is recognized by σ^H due to its time of activation based on the results of previous studies (Waalwijk et al., 1985; Ward and Ellar, 1986), it has little or no similarity to the consensus sequence [(A/G)(A/G/C)AGGA(A/T)(A/T)T–14bp–(A/C)GAAT] (Fig. 1). In addition, the increase in *cyt1Aa* expression toward the end of sporulation observed in our experiments does not correspond with the transitional expression of *spo0H* during sporulation initiation. Further studies to show that σ^E or σ^H binds to the BtIII promoter are required to conclusively characterize this promoter. However, we favor a model, based on the collective data described here, that BtIII is activated by σ^E.

The variability in transcript levels observed by recombinants with different combinations or independent BtI, BtII and BtIII promoters during sporulation (Fig. 3) is not unexpected as the availability and relative concentrations of σ factors can fluctuate to accommodate efficient and differential expression of genes throughout different phases of sporulation (de Hoon et al., 2010). Our data show that (i) maximal level of *cry1Aa* transcripts occurs in the presence of the BtI promoter (σ^E) in combination with BtII (σ^K) and BtIII (σ^E), (ii) BtI is the most robust promoter of the three, followed by BtII and BtIII, and (iii) there is a general correlation between transcript levels, and crystal size, and the amount of Cyt1Aa produced

per spore (Table 2; Fig. 3 and 4). Nevertheless, our data also indicates that the presence of BtIII is not required for maximal expression, as its absence in the other constructs does not significantly alter transcript levels (Fig. 3). Interestingly, however, the larger the size of the Cyt1Aa crystals in a strain, the fewer the number of sporulating cells, as assessed by the number of spores produced due to a fitness cost associated with making large crystals (Table 2). When the sizes of Cyt1A crystals produced by individual promoters were compared, BtI and BtIII produced, respectively, the largest and smallest. However, Cyt1A crystals produced by all three promoters were smaller than those produced by BtI-BtII, and showed no significant differences in size compared to those produced by BtII-BtIII, suggesting that there might be competition between BtI and BtIII. (Table 2). Consistent with this pattern, the BtIII promoter construct yielded the smallest Cyt1Aa crystals but more cells per unit medium (Table 2 and 3, Fig. 4), providing a level of Cyt1Aa synthesis per ml that was essentially similar to those obtained with the constructs containing other promoter combinations (Fig. 5). This result is consistent with previous reports showing that increases in endotoxin yields correlated with reduced spore counts, a phenomenon likely due to nutrient expenditure dedicated crystal synthesis rather than other cellular processes, including sporulation (Park et al., 1998; 1999). We do not have a specific reason for the observed decrease in viable spore counts. However, our results are in contrast with those reported by Barboza-Corona et al. (2014) in which a recombinant *B. thuringiensis* HD73 synthesizing a heterologous chitinase showed an increase in viable spore counts when compared with wild-type HD73. In their study, they suggested that the rapid nutrient depletion led to activation of Spo0A, a master regulator for entry into sporulation (Fujita and Losick, 2005). Regardless, the reliability of our results was confirmed through three separate experiments in which the overall discernible differences in crystal size among the three constructs were consistently observed by phase contrast and scanning electron microscopy.

As with *cry11Aa,* sequences in the 3'-UTR of *cyt1Aa* have the potential to form a secondary stem-loop structure that functions in transcription termination and mRNA stability (Waalwijk et al., 1985; Ward and Ellar, 1986; Agaisse and Lereclus, 1995), both of which could ultimately contribute to the overall accumulation of Cyt1Aa in *B. thuringiensis subsp. israelensis*. Using the method of Tinoco *et al.* (Ge, 1999), ΔG values of −27.6 kcal and −17.2 kcal were predicted for the 3'-UTR stem-loops of, respectively, *cyt1Aa* and *cry11Aa* (Fig. 8A). This suggested that *cyt1Aa*'s stem-loop was more stable than that of *cry11Aa*, and therefore contributed to the relatively higher Cyt1Aa yield when compared to that of Cry11Aa (Ward et al., 1986; Agaisse and Ellar, 1995; Ge, 1999). The 10% decrease in Cyt1Aa synthesis following deletion of the *cyt1Aa* terminator (Fig. 8B, C) confirmed this sequence plays a role, though apparently minor, in net Cyt1Aa synthesis, a role that could also be substituted by a theoretically weaker *cry11Aa* stem loop, with little or no decrease in yield of Cyt1Aa.

Finally, it is known that mechanisms have evolved in *B. thuringiensis* subsp. *israelensis* to aggregate the four mosquitocidal proteins (Cyt1Aa, Cry4Aa, Cry4Ba, Cry11Aa) into a parasporal body delimited by fibrous matrix of unknown composition. This results in a highly effective PB capable of killing sensitive dipteran larvae, i.e., it is much more toxic per unit weight than any of the endotoxins alone (Diaz-Mendoza et al., 2012). These toxins function synergistically, with the primary synergist being Cyt1Aa, which by itself is not very

toxic to mosquito larvae (Wirth et al., 1997). Previous studies have demonstrated that even at low levels Cyt1Aa is a potent synergist of Cry4Aa, Cry4Ab and Cry11Aa. Cyt1A can delay the evolution of resistance to mosquitocidal Cry proteins, for example, Cry11A, as well as the binary toxin of *Lysinibacillus sphaericus* (Wirth et al., Federici et al., 2007), and also restore the efficacy of these proteins against larvae resistant to them by allowing these toxins to bind to and/or pass through the midgut microvillar membrane (Federici et al., 2003). Thus, it might be possible to increase efficacy by reducing the amount of Cyt1Aa synthesized in *B. thuringiensis* subsp. *israelensis* to a level that retains its synergistic activity while increasing the amount of Cry and Bin proteins in recombinant bacteria. Such strains presumably could be more robust larvicides than wild-type or current recombinant strains (Federici et al., 2007). In this regard, our results reported here may assist in devising new strategies for genetically manipulating *cyt1Aa*, for example, by deleting the BtI and BtII promoters allowing for proportional and optimal increases of other mosquitocidal proteins that are assembled in the parasporal body.

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- Identifying the third promoter (BtIII) that has high identity to the σ^E promoter of *Bacillus subtilis*, from the 5'-untranslated region (UTR) of *cyt1Aa*
- BtIII is not functional in a σ^E-deficient strain of *B. subtilis* and is active throughout sporulation.
- Demonstrating that a stable stem-loop in the 3'-UTR (predicted $\Delta G = -27.6$) contributes to the high level of Cyt1Aa synthesized

A

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  1  TTTTCGATTT CAAATTTTCC AAACTTAAAT ATGATTGAAT GCCTGAGAAA

 51  GGTAATAGAG ATGTTTTAGT TTATTATGAA GTATTAGGGG CGTCTTTTAA

       T1▶ (-405)
101  ATTCAATCTA TCAATTTGTG AAATATATTA CTCAAAACCC AATACCATTC

151  TAAAACTTAT TCAAAATATA TATTGCTTTA AAAGAGCATA CATACTAAAA

               -35          BtI          -10     T2▶ (-271)
201  AAACAGGCAT CTTTCGAACT ATAGCGCATA GAATACTACG GTGAATCAAA

251  AACAAATAAA ATTTAGGAGG TATATTCAAG TATACAAAAA AACTTTAGTG

301  TGAGGGGATT TAGATAAAAA GTATTCGTTA TCCTTATAAA TTAATTCTTA

           -35          BtII          -10       T3▶ (-119)
351  AACATGCACC AATGTATACA TTAAATAATA TTATGTGAAT TAAGTCTATC

                                                -35       BtIII
401  AATTTAATTT ATTATGTTAC TTTATATTTG ATTAATAATT GCAAGTTTAA

        -10        T4▶ (-43)                              RBS
451  AATCATAATT TAATGTTGAA AGGCCACTAT TCTAATTAAC TTAAGGAGTT

             +1▶ (translation start)
501  GTTTATTTAT G
```

B

Sigma Factor	-35	Spacer (bp)	-10
cyt1A BtIII	TAATAAT	14	CATAATTT
σ^E	ZHATAXX	14	CATACAHT
σ^K	AC	17	CATANNNTA
σ^F	GCATR	15	GGHRARHTX
σ^G	GHATR	15	CATXHTA
σ^H	RNAGGAXXX	11-12	RNNGAAT

Fig. 1.

Functional *cis* elements in the 5'-untranslated region (5'-UTR) of *cyt1Aa*. **A.** Nucleotide sequence of the 5'-UTR of the *cyt1Aa* gene. The six known transcript start sites (T1, T2, T3, T4, T5, T6) identified in this and previous work are shown, as are the -35 and -10 sequences of BtI and BtII promoters (underlined), and the BtIII promoter (black background) identified in this study. The ribosome binding site (RBS, italicized) and Cyt1Aa translation initiation codon (=1, ATG) are also shown. **B.** Comparison of the *cyt1Aa* BtIII promoter region with that of the σ^E, σ^K, σ^F and σ^G regions of *B. subtilis*. H, A or C; N, A, G, C or T; R, A or G; X, A or T; Z, T or G.

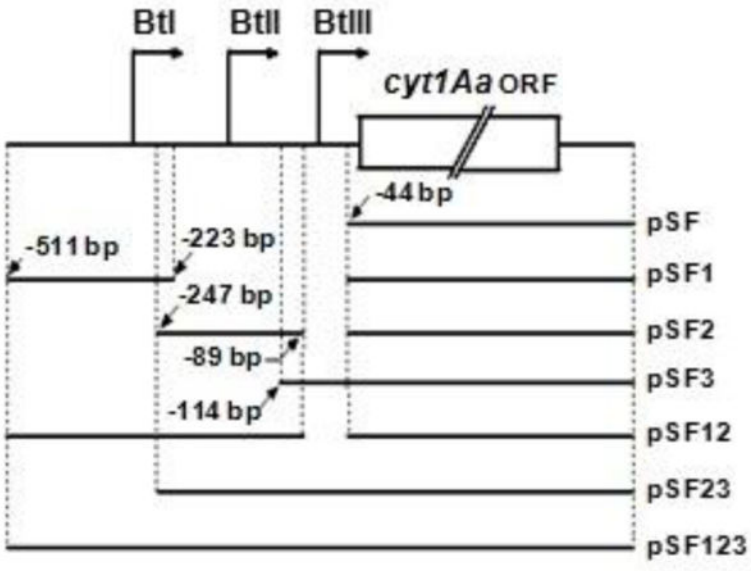

Fig. 2.

Schematic illustration of the seven *cyt1A* promoter constructs used to synthesize Cyt1Aa. The 5' starting and 3' ending points from the translational start site of the fragments used for each construct are indicated. The location of each *cyt1A* promoter (BtI, BtII and BtIII) is identified with an arrow.

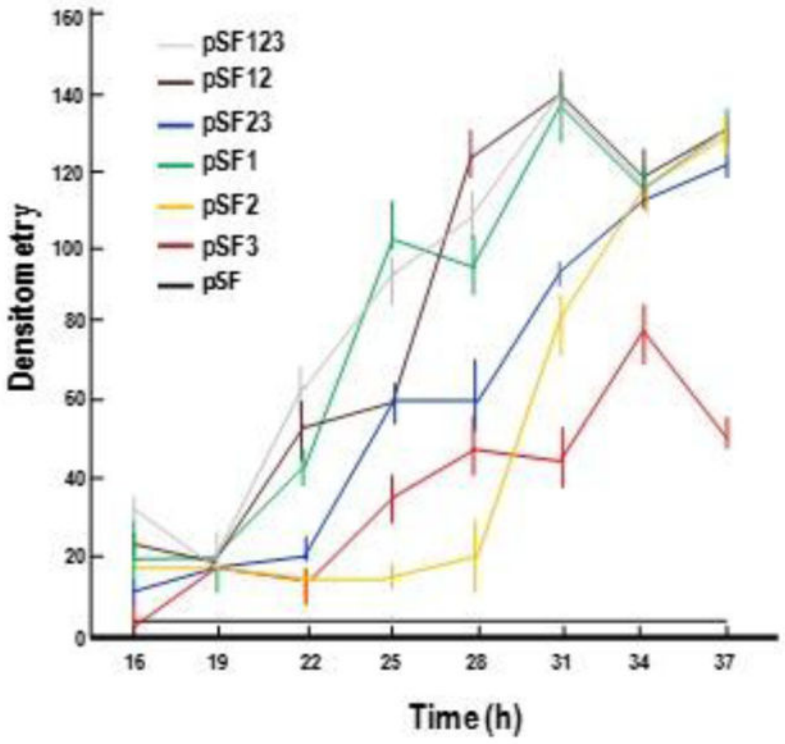

Fig. 3.

Densitometry of *cyt1Aa* transcripts from triplicate assays detected in various strains harboring different promoter (BtI, BtII, BtIII) constructs; pSF123 (BtI+II+III); pSF12 (BtI +II); pSF23 (BtII+III); pSF1 (BtI), pSF2 (BtII), pSF3 (BtIII); pSF (no promoter).

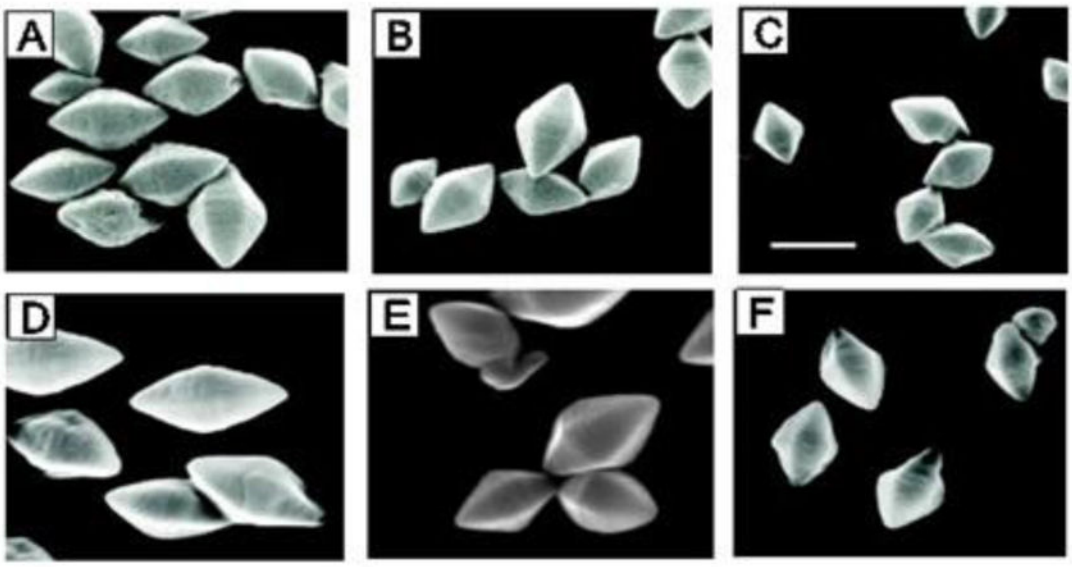

Fig. 4.

Scanning electron micrographs of Cyt1Aa crystals produced using the four different promoter constructs. A. Crystals produced using the 511 bp upstream sequence that contains all three promoters (BtI, BtII and BtIII; 4Q7/pSF123); B. Crystals produced using the 247 bp upstream sequence that contains only BtII and BtIII (4Q7/pSF23); C. Crystals produced using the 116 bp upstream sequence that contains only BtIII (4Q7/pSF3); D. Crystals produced using the 420 bp upstream sequence that contains only BtI and BtII (4Q7/pSF12); E. Crystals produced using the 286 bp upstream sequence that contains only BtI (4Q7/pSF1); F. Crystals produced using the 151 bp upstream sequence that contains only BtII (4Q7/pSF2). A bar indicates 1 μm and all micrographs are of the same magnification.

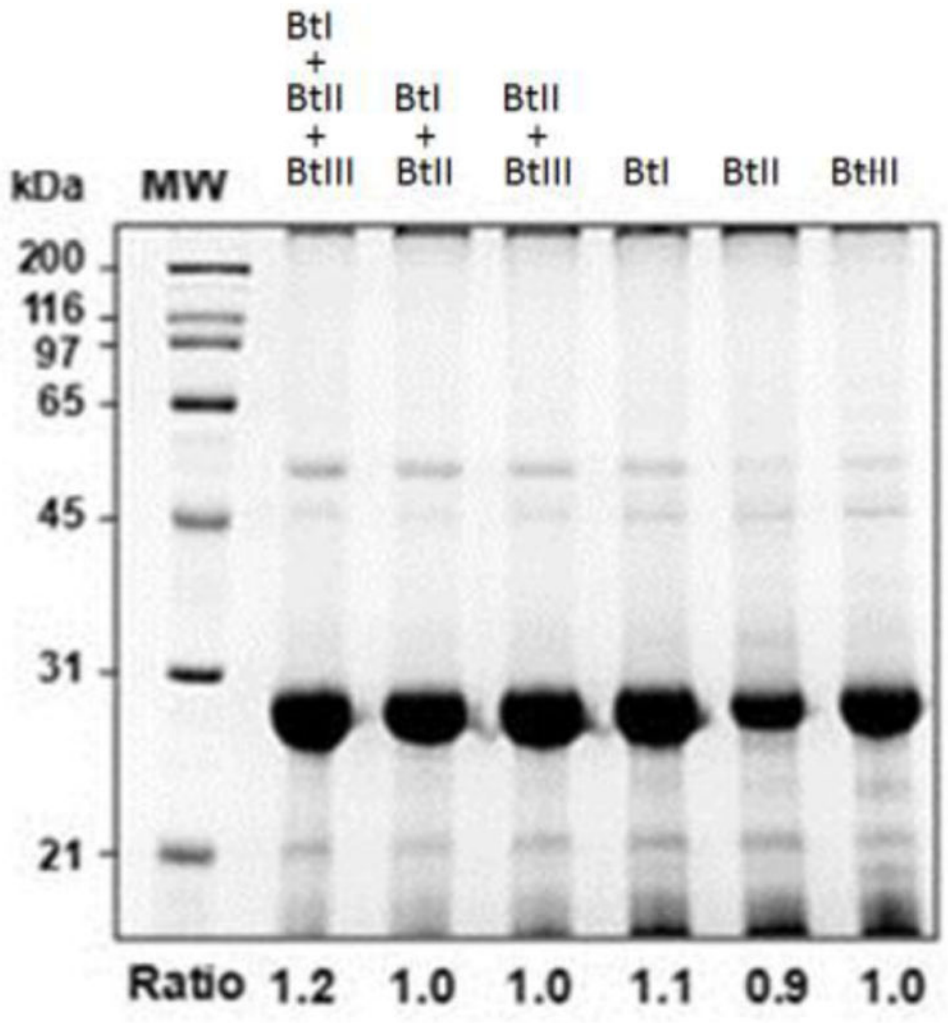

Fig. 5.

Analysis of Cyt1Aa protein synthesis by SDS-PAGE in *B. thuringiensis* using different promoter constructs. Lane M, protein markers; lane 1, Strain with the 511 bp upstream sequence that contains all three promoters (BtI, BtII and BtIII; 4Q7/pSF123); lane 2, Strain with 420bp upstream sequence that contains BtI and BtII (4Q7/pSF12); lane 3, Strain with the 247 bp upstream sequence that contains only BtII and BtIII (4Q7/pSF23); lane 4, Strain with the 286 bp upstream sequence that contains only BtI (4Q7/pSF1); lane 5, Strain with the 151 bp upstream sequence that contains only BtII (4Q7/pSF2); lane 6, Strain with the 116 bp upstream sequence that contains only BtIII (4Q7/pSF3). Relative ratio of Cyt1Aa production for each strain is shown at the bottom of the gel. This gel is a representative of results obtained in five separate replications.

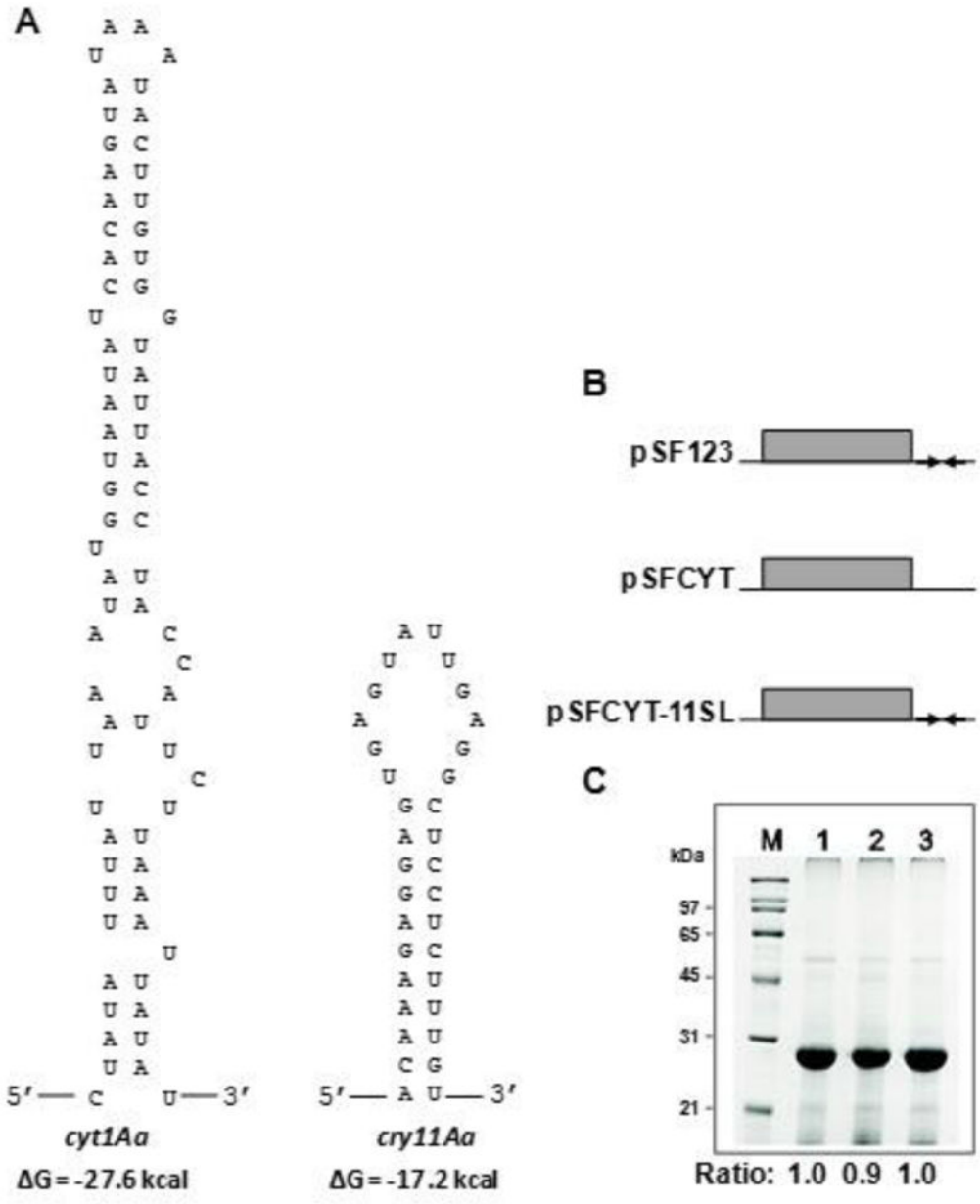

Fig. 6.

The -35 and -10 boxes are essential *cis* elements in BtIII for synthesis of Cyt1Aa. **(A)** Strains harboring cyt1Aa constructs with native -35 and -10 sequences (4Q7/SF3a), and nucleotide substitutions in -35 (4Q7/SF3μ35) and -10 (4Q7/SF3μ10). Recombinant strains were grown for 48 hr at which time >95% of cells sporulated and lysed and replicate culture samples were analyzed by SDS-PAGE **(B)** and Western blot analysis **(C)** for the presence of Cyt1Aa; purified Cyt1Aa (lane 1), 4Q7/SF3a (Cyt1Aa crystal observed by microscopy, lane 2), 4Q7/SF3μ35 (acrystalliferous, lanes 3, 4), 4Q7/SF3μ10 (acrystalliferous, lanes 5, 6); MW, molecular mass standards. Arrow, location of Cyt1Aa (top band) of the doublet.

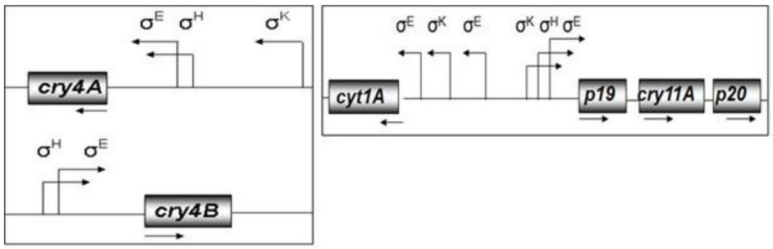

Fig. 7.

Analysis of Cyt1Aa synthesis in *B. subtilis* σ-deficient mutants transformed with pSF3 that contains the BtIII promoter for expression of *cyt1Aa*. **A.** SDS-PAGE analysis of *B. subtilis* strains grown in GYS medium. **B.** Western blot analysis of Panel A using antibody raised against Cyt1Aa. Lane M, protein markers; lane 1, 1S60 (σ^E mutant); lane 2, 1S60 with BtIII; lane 3, 1S38 (σ^K mutant); lane 4, 1S38 with BtIII; lane 5, 1S86 (σ^F mutant); lane 6, 1S86 with BtIII; lane 7, spoIIIGΔ1 (σ^G mutant); lane 8, spoIIIGΔ1 with BtIII; lane 9, *B. thuringiensis* 4Q7 harboring BtIII. Arrowhead indicates the 27-kDa Cyt1A protein band.

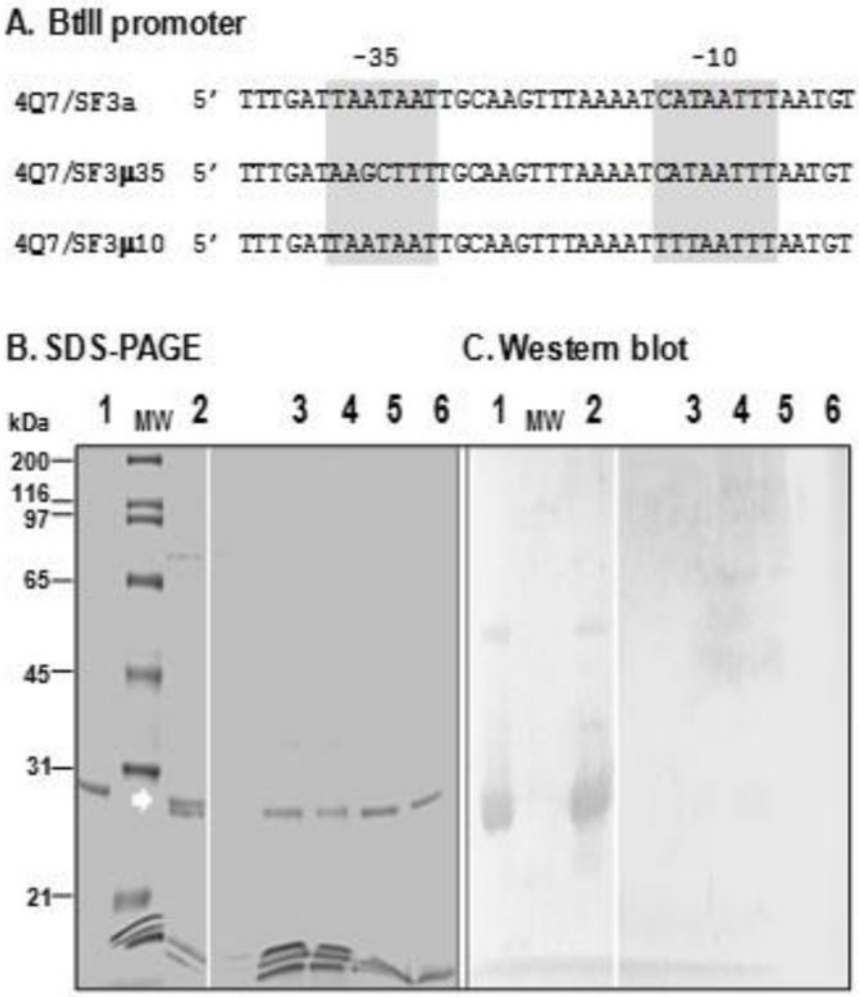

Fig. 8.

Effect of the 3’-untranslated region (3’-UTR) of the *cyt1Aa* gene on synthesis of Cyt1Aa in *B. thuringiensis*. **A.** Putative stem-loop structures formed at the 3’-ends of *cyt1Aa* and *cry11Aa* mRNAs, with a predicted ΔG value of -27.6 and -17.2 kcal as determined by the method of Tinoco *et al.* (Ge, 1999). **B.** Constructs used to test the effect of 3’-flanking regions of the *cyt1Aa* gene on the production of Cyt1Aa proteins in *B. thuringiensis* by replacing the *cyt1Aa* terminator with *cry11Aa* terminator. Plasmid pSFCYT, *cyt1Aa* gene lacking its terminator; pSF123, *cyt1Aa* gene with its own terminator and pSFCYT-11SL, *cyt1Aa* gene with native terminator replaced with the terminator from the *cry11As* gene. **C.** SDS-12% PAGE analysis of the relative level of Cyt1Aa proteins from the same volume of *B. thuringiensis* 4Q7 cell cultures with different constructs. Lane M, protein marker; lane 1, the strain harboring plasmid that has *cyt1Aa* with its own 3’ termination sequence [pSF123]; lane 2, the strain harboring plasmid that has *cry11Aa* without its own 3’ termination sequence [pSFCYT]; lane 3, the strain harboring plasmid that has *cyt1Aa* with the *cry11Aa* 3’ termination sequence [pSFCYT-11SL]. The rations at the bottom of the lanes were determined by densitometry scanning of the gel.

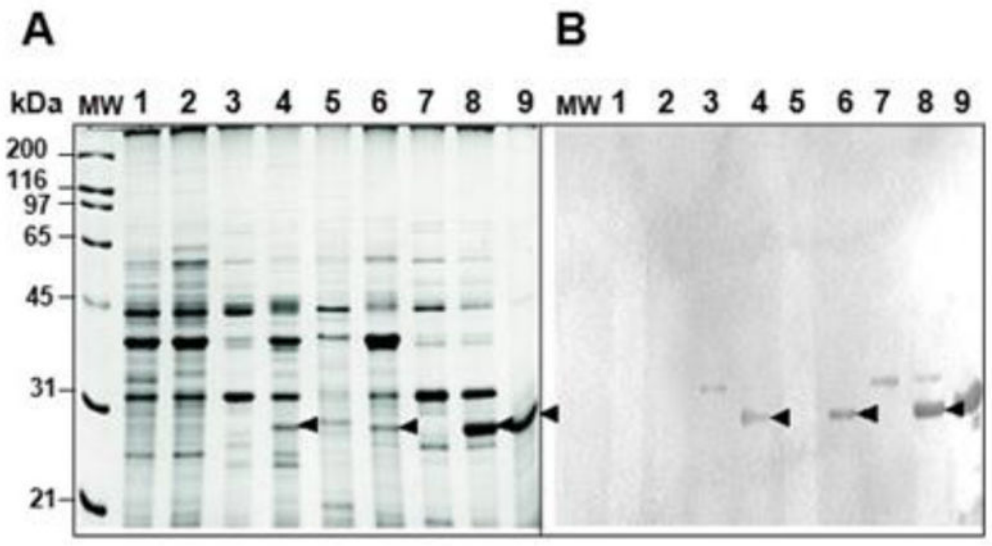

Fig. 9.

Schematic illustration of the sigma factors that recognize the promoters of *cyt1Aa*, *cry11Aa*, *cry4Aa* and *cry4Ba*. The sigma factors involved in the expression of endotoxin genes of *B. thuringiensis* subsp. *israelensis* and references to these are as follows: *cyt1Aa* (Waalwijk et al., 1985; Ward and Ellar, 1986; Ward et al., 1986; Brown and Whiteley, 1988), *cry11Aa* (Dervyn et al., 1995; Poncet et al., 1997), *cry4Aa* (Yoshisue et al., 1995; 1997), and *cry4Ba* (Poncet et al., 1997; Yoshisue et al., 1997).

Table 1

Primers used for amplifying genes in this study.[a]

Primer	Sequence (5’ to 3’)
CRY1Ac-P-F	acgcgtcgacGTTAACACCCTGGGTCAAAAATTGATA
CRY1Ac-P-R	ATCTCTTTTATTAAGATACCAATT
20 kDa-F	ATTGGAGGATAATTGATGACAGAA
20 kDa-R	aactgcagTTTAGGTCTTTAAAAATTAGAACCAA
CYT1-1-F	gctctagaTTTTCGATTTCAAATTTTCCAAACT
CYT1-2-F	gctctagaAGGAGGTATATTCAAGTATACA
CYT1-3-F	gctctagaCTATCAATTTAATTTATTATGTTAC
DBBT3	ggctctagaTTTGATTAATAATTGCAAGTTTAAAATCAT
DBBT3M	ggctctagaTTTGATAAGCTTTTGCAAGTTTAAAATCATAATTTAATGTTGAAAGGCCACTA
DBBT3TB	ggctctagaTTTGATTAATAATTGCAAGTTTAAAATTTTAATTTAATGTTGAAAGGCCACTA
CYT1-4-F	gctctagaGAAAGGCCACTATTCTAATTAACTT
CYT1-R	acgcgtcgacTCGAAAAATGTGGATGTGTGAAGAACA
CYT1-sigE-F	ggcgagctcTTTTCGATTTCAAATTTTCCAAACT
CYT1-sigE-R	gctctagaTGTATACTTGAATATACCTCC
CYT1-sigK-F	ggcgagctcAGGAGGTATATTCAAGTATACA
CYT1-sigK-R	gctctagaGTAACATAATAAATTAAATTGATAG
Cyt-nostem-R	acgcgtcgacTTAGAGGGTTCCATTAATAGCGCTAGT
Cyt-11Astem-R	gctctagaTTAGAGGGTTCCATTAATAGCGCTAGT
11Astem-F	gctctagaAAGTCATGTTAGCACAAGAGGAGTGA
11Astem-R	acgcgtcgacCATATGTTTTAAACAATTATTAGAA

[a]The restriction endonuclease sites were underlined: *Sal*I for CRY1Ac-P-F, *Pst*I for 20 kDa-R, *Xba*I for all CYT1-F, CYT1-sigE-R, CYT1-sigK-R, Cyt-11Astem-R, and 11Astem-F, *Sal*I for CYT1-R, Cyt1-nostem-R, and 11Atem-R, *Sac*I for CYT1-sigE-F and CYT1-sigK-F, and *Cla*I for SL-1 and SL-2.

Table 2

Dimensions and estimated volumes of Cyt1A crystals, mean number of spores produced per ml and estimated quantities of Cyt1A produced by different promoter constructs in *Bacillus thuringiensis*[a]

Promoter Combination[b]	Length ± SD µm	Width ± SD µm	Volume µm^3[c]	Spores/ml x 10^5 (± SD X 10^5)[d]	Relative Ratio	Cyt1A per ml of culture (±SD) (µg)[e]	Cyt1A per spore (± SD) (pg)[f]	Ratio[g]
BtI+BtII+BtIII	1.11 ± 0.10 *a*	0.65 ± 0.08 *d*	0.20 ± 0.07 *g*	20.2 (1.2) *a*	0.45	63.4 ± 1.1 *a*	31.4 ± 0.6 *f*	2.9
BtI+BtII	1.15 ± 0.14 a	0.81 ± 0.06 *e*	0.33 ± 0.09 h	9.3 (1.1) *b*	0.21	52.0 ±0.0 *b*	56.1 ± 0.0 *g*	5.2
BtII+BtIII	0.98 ± 0.15 *b*	0.63 ± 0.10 *d*	0.18 ± 0.08 *g*	21.1 (2.3) *a*	0.47	54.0 ±1.1 *c*	25.7 ± 0.5 h	2.4
BtI	1.08 ± 0.11 *a*	0.77 ± 0.08 *e*	0.28 ± 0.10 h	16.9 (0.6) *c*	0.37	54.5 ±0.9 *c*	32.3 ± 0.5 i	3.0
BtII	0.84 ± 0.10 *c*	0.58 ± 0.08 *f*	0.13 ± 0.05 i	31.8 (1.1) *d*	0.70	43.6 ± 0.4 *d*	13.7 ± 0.1j	1.3
BtIII	0.79 ± 0.13 *c*	0.54 ± 0.07 *f*	0.10 ± 0.04 j	45.2 (2.1) *e*	1.0	48.8 ± 0.9 *e*	10.8 ± 0.2 k	1.0

[a]Of a total of 45 to 105 crystal images per construct from the same batch culture, 20 crystals from each strain were selected for the measurement of sizes. Mean values for crystal dimensions were calculated separately (n = 20) from measurements made on scanning electron micrographs. Values followed by different letters were significantly different at P = 0.05.

[b]The names of the corresponding plasmids from the top to the bottom of the column are pSF123, pSF12, pSF23, pSF1, pSF2 and pSF3, respectively.

[c]The volume of crystals was estimated based on twice the volume of a regular hexagonal pyramid, which is calculated by V = 1/3 x area of hexagon x height. The width was used to calculate the area of the hexagon and the length was used for height.

[d]Mean values were determined based on three replicates. Values followed by different letters are significantly different at P = 0.05.

[e]Values were calculated based on the SDS-PAGE gel shown in Fig. 5 using a standard curve developed using series of diluted bovine serum albumin. Mean values were determined based on three replicates. Values followed by different letters are significantly different at P = 0.05.

[f]Values were calculated by dividing the quantity of Cyt1A produced per ml of culture in this table by the number of spores produced per ml of culture. Mean values were determined based on three replicates. Values followed by different letters are significantly different at P = 0.05

[g]The relative quantity of Cyt1A per spore was calculated giving the amount produced by 4Q7/pSF3 an arbitrary value of 1.