Identification of a Catabolite-Responsive Element Necessary for Regulation of the *cry4A* Gene of *Bacillus thuringiensis* subsp. *israelensis*[▽][†]

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Bacillus thuringiensis subsp. israelensis produces a potent mosquitocidal protein, Cry4A. We have identified a 15-bp catabolite responsive element (*cre*), overlapping the −35 element of the *cry4A* promoter. Changing a guanine to adenine at position −49 in the promoter abolished glucose catabolite repression of *cry4A* and enhanced promoter activity two- to threefold. This *cis* regulatory element is essential for controlled toxin synthesis, vital to evolutionary success of B. thuringiensis subsp. israelensis.

Bacillus thuringiensis produces insecticidal crystal proteins highly toxic to insects (6, 14). Synthesis of insecticidal proteins by *B. thuringiensis* is transcriptionally linked to sporulation through common transcription factors (3). Transcription of many stationary-phase genes, including *cry* genes, is held in check until effectors regulating the developmental process are induced (1). The developmental process in *B. thuringiensis* is temporally controlled at transcription level by recruitment of successive sigma subunits of the RNA polymerase (RNAP) (13). The following five σ factors are involved in the developmental process of *B. thuringiensis*: σ^H, which functions in the stationary phase in the undivided mother cell; σ^{35} and σ^{28}, which control early and late sporulation genes and most of the crystal protein genes in the mother cell; and σ^F and σ^G, which are active in the forespore compartment of the sporulating bacterium (25).

Toxin production in *B. thuringiensis* is subject to a variety of sophisticated controls at various levels to maximize protein yields (1, 6). *B. thuringiensis* subsp. *israelensis* produces an ovoid inclusion toxic to dipteran insects: e.g., mosquitoes and blackflies (10). The parasporal crystals are composed of a number of polypeptides, namely, Cry4A, Cry4B, Cry11A, and CytA (15). The *cry4A* gene encodes a 130-kDa protoxin protein, expressed weakly in the transition phase through σ^H-specific RNAP (27), while major amount of the protein is synthesized in the stationary phase under the control of σ^{35}- and σ^{28}-specific RNAP (33, 34).

Our earlier investigations probing the role of environmental factors regulating Cry4A expression in *B. thuringiensis* subsp. *israelensis* (5) demonstrated that glucose and its metabolite fructose 6-phosphate repressed *cry4A* gene expression through catabolite repression (CR) (16), a global, negative regulatory mechanism of gene transcription in gram-positive bacteria (11). CR establishes priorities in the utilization of carbon and energy sources according to their selective advantage (11) for competitive success of the bacteria in the natural environment. In gram-positive bacteria, the essential components of CR are catabolite control protein CcpA, a member of LacI/GalR family of bacterial regulatory proteins (31); an allosteric effector protein, HPr (4); and a *cis*-acting, partially palindromic binding sequence, the catabolite responsive element (*cre*) in the gene (9, 30).

Glucose repression of *cry4A* transcription in *B. thuringiensis* subsp. *israelensis* (5) and its derepression in an HPr-serine phosphorylation (Hpr-Ser-P)-defective mutant (16) suggested catabolite repression of the δ-endotoxin gene. To get an insight into the mechanism of *cry4A* repression, here we analyzed the *cry4A* gene and its upstream regulatory sequence for potential regulatory elements. We present data characterizing interaction of different components of the catabolite repression network with the *cry4A* gene leading to identification of a *cre*.

Search for *cre*-like sequence in the *cry4A* gene and upstream promoter region. Based on the 14-nucleotide consensus sequence TGWAANCGNTNWCA, proposed by Weickert and Chambliss (30), a pattern-matching search was performed in the *cry4A* gene and its flanking regions of *B. thuringiensis* subsp. *israelensis* strain ATCC 522. Four sequences matching the consensus *cre* sequence were identified in the latter (Fig. 1). Two sequences, *cre1* and *cre2* were present in the promoter region, 50 and 21 bases upstream of the transcriptional start site, showing 85.7% and 57.14% sequence similarity, respectively. *cre3* and *cre4* were present in the coding region of the gene and showed 71.43% and 78.57% similarity, respectively, to the consensus sequence.

Interaction of CcpA and HPr-Ser-P with identified *cre* sequences. The bacterial strains and plasmids used in this study are listed in Table 1, and the oligonucleotide primers are shown in a table in the supplemental material.

Protein DNA interactions were evaluated by mobility shift assays. A 117-bp probe DNA (−94 to +23) containing *cre1* and *cre2* (Fig. 1) was amplified by PCR with primers 3 and 4 using a *B. thuringiensis* subsp. *israelensis* megaplasmid as a template and cloned in pGEM-T Easy vector, producing pSK3. The $G_{49} \rightarrow A$ mutation was obtained using $G_{49} \rightarrow A$ forward and
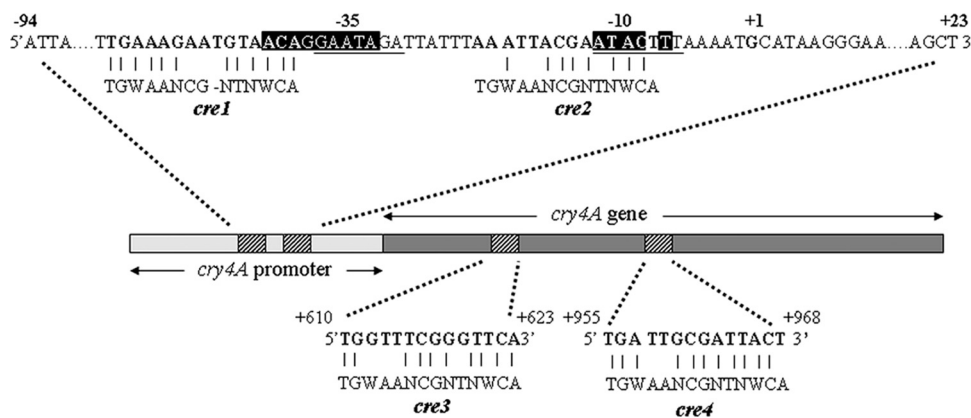
FIG. 1. Identification of *cre* sequences in the *cry4A* gene. Shown is the *cry4A* gene, with four *cre*-like sequences (*cre1*, *cre2*, *cre3*, and *cre4*) homologous to consensus sequence TGWAANCGNTNWCA. +1 represents the *cry4A* transcription initiation nucleotide, σ^{35}-recognized −35 and −10 elements are underlined, and the SigH promoter is shaded.

reverse primers with pSK3 as a template, producing plasmid pSK4. The plasmids pSK3 and pSK4 were digested with SpeI and EcoRI, and the 117-bp inserts were end labeled in the presence of $[\alpha\text{-}^{32}\text{P}]$dCTP (Perkin-Elmer), deoxynucleotide triphosphates (dNTPs), and the Klenow fragment of DNA polymerase for use as a probe. The probe DNA was purified on a Sephadex G-25 column (Pharmacia).

Double-stranded oligonucleotide fragments encompassing *cre3* (+594 to +651) and *cre4* (+931 to +986) were generated by annealing long complementary primer sequences 5 with 6 and 7 with 8 in annealing buffer ($1\times$ Tris-EDTA buffer [pH 8.0] and 50 mM NaCl). The reaction mixture was heated to 80°C and allowed to cool slowly to 25°C, and the 3′ overhangs were radiolabeled by end-filling reaction.

Recombinant CcpA and HPr kinase were prepared as described earlier (7, 12). HPr from *B. thuringiensis* subsp. *israelensis* was phosphorylated at Ser-45 using HPr kinase and purified as described earlier (17). The binding reaction mixture contained labeled probe (20,000 cpm/μl) with 0.6 nM HPr and 0.78 nM CcpA, 2 μg poly(dI-dC) (Pharmacia), 15 mM HEPES (pH 7.5), 35 mM KCl, 1 mM EDTA (pH 7.5), 1 mM dithiothreitol, 6% glycerol, and 1 mM MgCl$_2$ in a total volume of 45 μl. The proteins were incubated with poly(dI-dC) in the reaction buffer for 5 min at room temperature, followed by addition of 1.5 μl of probe DNA. The reaction mixture was incubated at 37°C for 20 min and loaded on a 5% nondenaturing polyacrylamide gel in $0.5\times$ Tris-borate-EDTA (TBE) buffer. Electrophoresis was conducted at 4°C for 1 h. The gel was dried and exposed to a PhosphorImager screen for 2 h, and the autoradiograms were analyzed with a Typhoon 9210 PhosphorImager (Amersham).

Binding of the proteins CcpA and HPr-Ser-P was tested with all four putative *cre* sequences. HPr or HPr-Ser-P alone showed no shift with any of the selected *cre* sequences (Fig. 2A, E, and F). CcpA alone retarded mobility of the DNA containing *cre1* and *cre2* (*cre1-2*) (Fig. 2A, lane 1), but large excess (400-fold) of cold, specific DNA (Fig. 2B) or 4 μg of nonspecific poly(dI-dC) DNA could not inhibit binding competitively, indicating the nonspecific nature of the interaction. Miwa et al. have also reported such nonspecific CcpA interaction earlier (24). Incubation of *cre1-2* with HPr-Ser-P and CcpA together

reduced the mobility of DNA in a concentration-dependent manner (Fig. 2C), although addition of HPr-Ser-P to CcpA produced only a marginal increase in mobility shift over CcpA alone. This could be due to the smaller size of HPr (runs like a 10-kDa protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis), compounded with an additional negative charge on HPr-Ser-P, pulling the complex more toward anode. The mobility shift of *cre1-2* with CcpA-HPr-Ser-P complex was specific, as a 200-fold excess of cold DNA abolished the binding completely (Fig. 2D). These results indicate that formation of CcpA-HPr-Ser-P complex in solution is conducive to DNA binding. These observations are supported by the structural study (29) showing significant local structural changes in the DNA binding domain of CcpA induced directly by binding of HPr-Ser-P. No shift in mobility of *cre3* or *cre4* DNA was observed when CcpA, HPr, and HPr-Ser-P alone or as complex were tested (Fig. 2E and F), excluding their involvement in regulation of the *cry4A* gene.

Identification of CcpA-HPr-P complex binding site on *cre1-2* by DNase I footprinting assay. To identify the precise binding sequence of *cre1-2*, DNase I footprinting was performed as described by Schmitz and Galas (28) with minor modification. The 117-bp fragment containing *cre1* and *cre2* was end labeled with $[\alpha\text{-}^{32}\text{P}]$dCTP at the SpeI end of the coding strand. The wild-type DNA or the $G_2\rightarrow A$ altered variant (20,000 cpm/μl) was incubated with CcpA and HPr-Ser-P proteins under conditions described above for gel retardation, except no EDTA was added to the buffer and each reaction mixture contained 2 μg poly(dI-dC) in a total volume of 45 μl. One unit of DNase I (Sigma) was added to the reaction mixture and incubated at room temperature for 2 min. The DNase I digestion was stopped by adding phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was precipitated with ethanol in the presence of 6 μg glycogen. The DNA was resolved in a 10% urea–polyacrylamide sequencing gel. A base-specific chemical cleavage sequencing reaction (21) was also performed in parallel on the same gel. The gels were dried and exposed to a PhosphorImager screen overnight.

No protection was observed when CcpA or HPr-Ser-P alone was used (Fig. 3A). However, binding of the CcpA-HPr-Ser-P complex protected the *cre1* region extending from position −50 to

TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Relevant characteristic(s)	Source or reference
Plasmids		
pGEM-T Easy	Amp^r, *E. coli* cloning vector	Promega
pHT370	Amp^r Ery^r, shuttle vector	Pasteur Institute
pSK1	pGEM-T Easy containing *cry4A* coding sequence with native promoter	This work
pSK2	pHT370 containing *cry4A* coding sequence with native promoter	This work
pSK3	pGEM-T Easy containing 117-bp *cre1-2*	This work
pSK4	pGEM-T Easy containing *cre1-2* with mutation ($G_{49} \rightarrow A$) in *cre1*	This work
pSK5	pGEM-T Easy containing 458-bp *cry4A* promoter	This work
pSK6	pGEM-T Easy containing 3,067-bp *lacZ*	This work
pSK7	pGEM-T Easy containing $cry4A_{pro}$-*lacZ* fusion	This work
pSK8	pHT370 containing $cry4A_{pro}$-*lacZ* fusion	This work
pSK9	pHT370 with *cry4A* coding sequence with $G_{49} \rightarrow A$ in *cre1*	This work
Strains		
4Q7	*B. thuringiensis* subsp. *israelensis* plasmidless mutant	BGSC[a]
SK2	4Q7 containing pSK2	This work
SK9	4Q7 containing pSK9 [($G_{49} \rightarrow A$) mutation in *cre1* in pSK2]	This work
SK10	4Q7 containing pSK8	This work
SK11	4Q7 containing pSK11 [($A_{36} \rightarrow C$) in pSK8][b]	This work
SK12	4Q7 containing pSK12 [($C_{37} \rightarrow A$) in pSK8][b]	This work
SK13	4Q7 containing pSK13 [($T_{40} \rightarrow C$) in pSK8][b]	This work
SK14	4Q7 containing pSK14 [($A_{43} \rightarrow G$) in pSK8][b]	This work
SK15	4Q7 containing pSK15 [($A_{44} \rightarrow C$) in pSK8][b]	This work
SK16	4Q7 containing pSK16 [($A_{47} \rightarrow C$) in pSK8][b]	This work
SK17	4Q7 containing pSK17 [($A_{48} \rightarrow C$) in pSK8][b]	This work
SK18	4Q7 containing pSK18 [($G_{49} \rightarrow A$) in pSK8][b]	This work
SK19	4Q7 containing pSK19 [($G_{49} \rightarrow T$) in pSK8][b]	This work
SK20	4Q7 containing pSK20 [($G_{49} \rightarrow C$) in pSK8][b]	This work

[a] BGSC, *Bacillus* Genetic Stock Center (Ohio State University, Columbus).
[b] Point mutations are done in the *cre1* region of the promoter.

−29 (23 bp) from DNase I digestion (Fig. 3B, lanes 2 and 3). The protected region overlapped with the −35 element of the σ^{35}-driven promoter of *cry4A* and showed 86% similarity to the 14-bp consensus sequence proposed by Weickert and Chambliss (30).

Mutational analysis of the DNase I-protected sequence identified G_{49} as the critical nucleotide base (results described in the following section), which was subsequently verified by footprinting also. The 117-bp variant with $G_{49} \rightarrow A$ mutation in the *cre1* region showed no protection from DNase I in the presence of CcpA-HP-Ser-P complex (Fig. 3C, lanes 2, 3, 4, and 5), corroborating the promoter activity results.

Evaluation of promoter activity in $cry4A_{pro}$-lacZ transcriptional fusions. The *cry4A* promoter ($cry4A_{pro}$) (458 bp upstream of the start codon) was amplified by PCR using primers 9 and 10 with KpnI and BamHI sites, respectively, with *B. thuringiensis* subsp. *israelensis* plasmid DNA. The coding region of the *lacZ* gene (3,067 bp) was obtained from *Escherichia coli* K-12 genomic DNA using primers 11 and 12 with BamHI and HindIII sites, respectively. The amplified DNA fragments were cloned into pGEM-T Easy vector, giving pSK5 and pSK6,

respectively. The *cry4A-lacZ* fusion (pSK7) was produced by excising $cry4A_{pro}$ from pSK5 with NcoI/BamHI and ligating it to *lacZ* sequence in pSK6 at complementary sites. The promoter-*lacZ* fusion was excised from pSK7 with KpnI/HindIII and ligated in pHT370, an *E. coli-Bacillus* shuttle vector (Pasteur Institute), resulting in reporter construct pSK8.

To map the *cre1* region revealed by footprinting, we performed base exchange mutagenesis using the Quik Change site-directed mutagenesis kit (Stratagene). Plasmids pSK8 (wild-type $cry4A_{pro}$-*lacZ* gene fusion) and pSK2 (wild-type *cry4A* gene with promoter) were used as template for mutations with primers listed in the table in the supplemental material. The resulting plasmids with designated mutations in the *cre1* region are listed in Table 1. A crystal-negative *B. thuringiensis* subsp. *israelensis* strain, 4Q7, obtained from the *Bacillus* Genetic Stock Center (Columbus, OH) was electroporated with the plasmids by the protocol described by Macaluso and Mettus (19). Two hundred microliters of competent cells was electroporated with 1 to 5 μl of plasmid DNA at 2.5 kV, 200 Ω, and 25-μF capacitance. The transformants were selected on Luria-Bertani plates containing 25 μg of erythromycin/ml.

Cells of strain 4Q7 containing the promoter variants were grown in 50 ml G medium (2) containing G salts, 0.2% glucose, and 0.15% yeast extract with erythromycin for 10 h (optical density at 595 nm [OD_{595}] of 1.5), 14 h (OD_{595} of 1.8) or 18 h
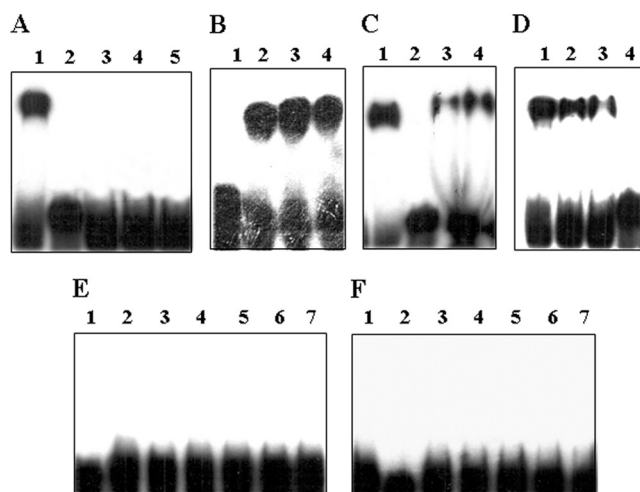

FIG. 2. (A) Binding of CcpA, HPr, and HPr-Ser-P to 117-bp probe containing *cre1-2* by electrophoretic mobility shift assay. Lane 1, 0.78 nM CcpA; lane 2, free probe; lane 3, 0.6 nM HPr; lanes 4 and 5, 0.6 nM and 1.2 nM HPr-Ser-P, respectively. (B) Binding of CcpA to 117-bp probe and competition with cold DNA. Lane 1, free probe; lane 2, 0.78 nM CcpA; lanes 3 and 4, 0.78 nM CcpA plus 200- and 400-fold excess cold probe DNA, respectively. (C) Binding of CcpA and HPr-Ser-P to 117-bp probe. Lane 1, 0.78 nM CcpA; lane 2, free probe; lanes 3 and 4, 0.78 nM CcpA plus 0.6 nM and 1.2 nM HPr-Ser-P, respectively. (D) Competition of CcpA-HPr-Ser-P complex binding to 117-bp probe with cold probe DNA. Lane 1, 0.78 nM CcpA plus 0.6 nM HPr-Ser-P; lanes 2, 3, and 4, 0.78 nM CcpA plus 0.6 nM HPr-Ser-P with 50-, 100-, and 200-fold excess cold competitor DNA, respectively. (E) Binding of CcpA, HPr, and HPr-Ser-P to probe DNA containing *cre3*. Lane 1, free probe; lane 2, 0.78 nM CcpA; lane 3, 0.6 nM HPr; lanes 4 and 5, 0.6 nM and 1.2 nM HPr-Ser-P, respectively; lanes 6 and 7, 0.78 nM CcpA plus 0.6 nM and 1.2 nM HPr-Ser-P, respectively. (F) Binding of CcpA, HPr, and HPr-Ser-P to probe DNA containing *cre4*. The lanes are as in panel E.
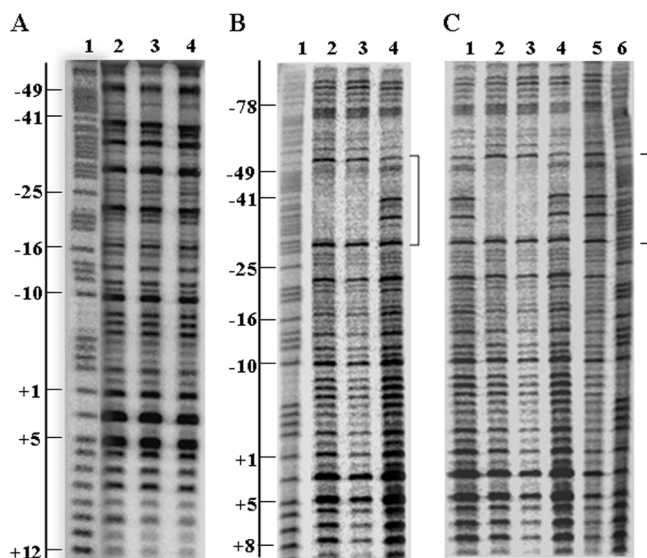
FIG. 3. DNase I footprinting analysis of CcpA and HPr-Ser-P binding to the *cre1* in the 117-bp fragment of the *cry4A* promoter. (A) Lane 1, G+A marker; lane 2, no-protein control; lane 3, 0.78 nM CcpA; lane 4, 0.6 nM HPr-Ser-P. Nucleotide positions (with +1 representing the transcription initiation nucleotide) are given on the left. (B) Protection of 117-bp wild-type DNA with CcpA-HPr-Ser-P complex. Lane 1, G+A marker; lanes 2 and 3, 0.78 nM CcpA plus 0.6 nM and 1.2 nM HPr-Ser-P, respectively; lane 4, no-protein control. The protected region is indicated on the right by a bracket in panels B and C. (C) DNase I protection of 117-bp wild-type and $G_{49} \rightarrow A$ variant promoter DNA. Lane 1, no-protein control; lanes 2 and 3, 117-bp wild-type DNA with 0.78 nM CcpA plus 0.6 nM and 1.2 nM HPr-Ser-P, respectively; lanes 4 and 5, 117-bp $G_{49} \rightarrow A$ DNA with 0.78 nM CcpA plus 0.6 nM and 1.2 nM HPr-Ser-P, respectively; lane 6, G+A marker.

(OD_{595} of 1.75). To induce Cry4A synthesis, the cells were harvested, washed, and suspended in a resuspension medium (RM) [50 mM Tris-HCl [pH 7.5], 0.008% $CaCl_2 \cdot 2H_2O$, 0.05% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.00005% $FeSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.005% $MnSO_4 \cdot H_2O$, 0.02% $MgSO_4$], at 25 ml each in the presence of 0.6% glucose or no glucose. The cultures were incubated at 29°C with shaking, 1-ml aliquots were withdrawn every 2 h up to 8 h, and OD_{595} was measured. The cells were centrifuged, washed with ice-cold 25 mM Tris-HCl (pH 7.5), and suspended in 0.8 ml buffer Z (100 mM sodium phosphate buffer [pH 7.5], 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) containing 0.4 mg lysozyme for permeabilization. The specific activity of β-galactosidase was determined using *O*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) as described earlier (22). One unit of β-galactosidase is the amount of enzyme producing 1 nmol of *O*-nitrophenol per min at 30°C. The average of triplicate samples was determined. The experiment was performed more than three times, and the data from one representative experiment are presented.

The mutations were selected to substitute most conserved bases in the consensus sequence proposed by Weickert and Chambliss (30). The basic principle was to introduced transversions from AT to CG and vice versa. A progressive decrease in glucose repression occurred as the position of the mutation moved across the *cre* from 3′ to 5′ and was abolished at $G_{49} \rightarrow A$, the putative σ factor contact site (18). The substitu-

tions $G_{49} \rightarrow T$ and $G_{49} \rightarrow C$ also reduced the repression ratio, but not as dramatically as $G_{49} \rightarrow A$ (Fig. 4). Mutations of $A_{44} \rightarrow C$ and $A_{43} \rightarrow G$ were performed to create the conserved CG at the center. Contrary to expectations, the mutations resulted in partial relief from glucose repression instead of enhancing it (data not shown). A second possibility of replacement of A_{43} and T_{42} with CG was not attempted, so its positive effect on repression cannot be ruled out. The centrally placed CG is considered highly conserved, and mutations of these bases are reported to result in loss in activity of the *cre* (9, 30, 32). However, like the *cry4A* gene, in several instances their absence is of little or no consequence to the activity (23). The substitution $A_{48} \rightarrow C$ close to G_{49} also caused substantial relief from glucose repression. Although mutation of the other conserved positions at the 3′ end of the *cre*, namely $A_{36} \rightarrow C$, resulted in low repression ratio, the promoter activity was also reduced below the wild-type level (Fig. 4). The above results establish the presence of a 15-bp *cre* element in the *cry4A* gene which although 1 base longer shows strong similarity to the commonly observed 14-bp consensus sequence, although a longer (16 bp) *cre*, associated with the *lev* operon, has also been reported earlier in *B. subtilis* (20). The identified *cre* sequence overlaps the −35 element partially and extends up to the −50 position on the promoter. Apparently the CcpA-HPr-Ser-P complex blocks the RNAP recognition site on the promoter, inhibiting transcription (26).

Promoter activity was low in cells containing the wild-type promoter grown for 10 h, and no glucose repression occurred. The promoter activity increased in both stationary-phase (14 h) and post-stationary-phase (18 h) cells and was highest in the latter, perhaps due to transcription from two different σ^{35} and σ^{28} promoters, known to occur commonly in other *cry* genes (6, 33). Parallel to the increase in transcriptional activity, glucose repression was also observed in the cells grown for 14 h (88%) and 18 h (55%) (Fig. 4). The repression was relieved significantly by $G_{49} \rightarrow A$ mutation in both cells grown for 14 h (93%) and 18 h (89%). The results reveal that glucose repression of *cry4A* lasts until late in the post-stationary phase (18-h grown), when most of the glucose is consumed; hence, a glucose metabolite such as fructose-1,6-bisphosphate or one whose identity is not known at the moment might be mediating the glucose effect in the later phase.

Interestingly, the promoter activity also increased approximately threefold by $G_{49} \rightarrow A$ mutation, (Fig. 4), suggesting more efficient binding/transcription of the promoter by RNAP. An explanation that needs to be verified experimentally could be generation of a sequence resembling the UP element, the A+T-rich component of bacterial promoters that stimulates transcription by interacting with the α subunit of RNAP (8). The RNAP holoenzyme in its extended form covers 70 to 80 bp from −55 to +20 of DNA, while the σ factor, which initiates recognition, covers the region from −55 to −35 (18). In the light of the above facts, a more efficient recognition/initiation of the promoter by the σ or α subunit of RNAP may result in elevated promoter activity. Thus, the vital role of guanine at −49 in orchestrating toxin gene regulation through twin effects on promoter activity is the key finding of this study and could be useful in developing a high-toxin-yielding strain.

Evaluation of *cry4A* gene expression by semiquantitative RT-PCR and Western blotting. The *cry4A* gene with the promoter region (4001 bp) was amplified from *B. thuringiensis*
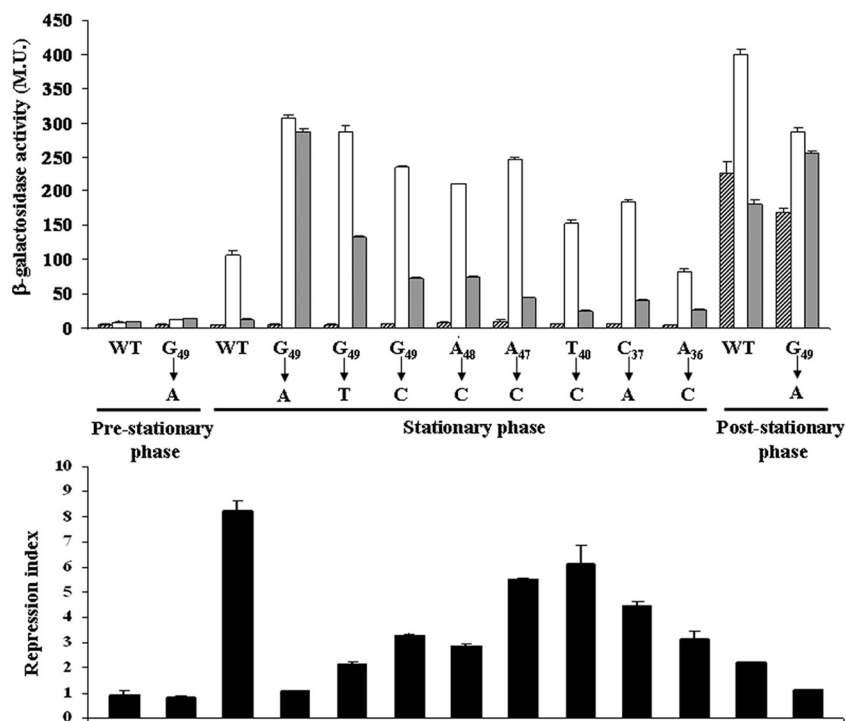
FIG. 4. Effect of glucose on *cry4A* promoter activity in vivo during different growth phases. *cry4A* promoter-*lacZ* fusion variants were grown in G medium (8 h for pre-stationary phase, 14 h for stationary phase, and 18 h for post-stationary phase) and resuspended in RM (upper panel) with glucose (gray bars) or without glucose (white bars). β-Galactosidase activities were measured at 8 h after resuspension. The hatched bars (T_0) show β-galactosidase activities at the time of resuspension in RM. WT, wild type. The repression index (lower panel) was calculated as the ratio of β-galactosidase activity obtained in the absence of glucose to that in the presence of glucose. Subscripts show nucleotide positions relative to +1, and substitutions are shown with arrows. Values are the average of three independent experiments, each assayed in duplicate. Error bars represent the standard deviation. M.U., Miller units.

subsp. *israelensis* plasmid, with forward primer 1 and backward primer 2. The amplified DNA fragment was cloned in the pGEM-T Easy vector (Promega), producing pSK1. The gene was ligated in the pHT370 vector, and pSK2 was obtained; $G_{49} \rightarrow A$ mutation was performed in pSK2, producing pSK9.
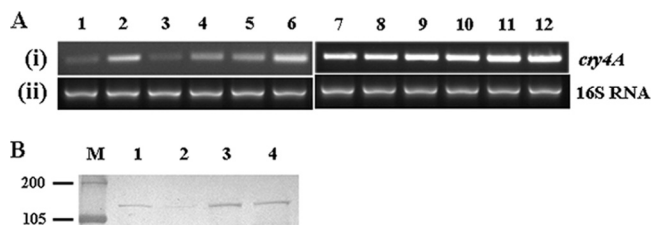

FIG. 5. (A) Evaluation of glucose effect on *cry4A* transcription. Cells of strain 4Q7 were grown in G medium for 14 h and resuspended in RM with or without glucose. Samples were collected at different time points after resuspension in RM. (i) *cry4A* transcripts in the SK2 cells, lanes 1 to 6. Lanes 1, 3, and 5, with glucose after 1, 2, and 3 h, respectively; lanes 2, 4, and 6, no glucose after 1, 2, and 3 h, respectively. Lanes 7 to 12 contained *cry4A* transcripts in SK9 cells. Lanes 7, 9, and 11, with glucose after 1, 2, and 3 h, respectively; lanes 8, 10, and 12, no glucose after 1, 2, and 3 h, respectively (ii) 16S RNA loading control of respective test samples. (B) Western blot of Cry4A expression in 4Q7 strains. The SK2 and SK9 strains were grown in G medium and resuspended in RM, and samples were taken at 24 h. Lanes 1 and 2, total protein from SK2 cells without and with glucose, respectively; lanes 3 and 4, total protein from the SK9 ($G_{49} \rightarrow A$) variant without and with glucose, respectively.

Both plasmids were transformed in strain 4Q7 (Table 1), producing SK2 and SK9.

The SK2 (*cry4A* with wild type promoter) and SK9 (*cry4A* with $G_{49} \rightarrow A$ variant promoter) strains were grown in G medium for ~14 h and resuspended in RM as described above, with or without 0.6% glucose. Total RNA was isolated from 5 ml culture using the RNeasy minikit (Qiagen) and treated with DNase I. About 0.3 μg of DNA-free total RNA was used as the template in reverse transcription-PCR (RT-PCR) using the One Step RT-PCR kit (Qiagen). Primer 14 and internal reverse primer 15 were used to amplify a 414-bp fragment of the *cry4A* gene. A 16S RNA gene fragment was amplified using primers 16 and 17 as a loading control. The PCR conditions were 94°C denaturation for 45 s, 54°C annealing for 45 s, and 72°C extension for 30 s for *cry4A* and 1.5 min for 16S RNA.

Glucose repressed *cry4A* transcripts in cells containing the wild-type construct at 1, 2, and 3 h after resuspension (Fig. 5A, lanes 1 to 6), while no effect was observed in the $G_{49} \rightarrow A$ variant (Fig. 5A, lanes 7 to 12). At all time points, concentrations of *cry4A* transcripts were two- to threefold higher in the G_{49} mutant than the wild-type strain, implying increased promoter activity.

Total proteins were isolated from SK2 and SK9 cells grown as described above for RNA isolation. Two-milliliter samples were removed at different intervals, and the cell pellets were solubilized in sodium dodecyl sulfate-polyacrylamide gel elec-

trophoresis loading dye. Proteins were resolved by electrophoresis and transferred on nitrocellulose membrane by standard methods, and expression of Cry4A protein was detected using polyclonal antiserum diluted 1:10,000. The antiserum against recombinant domain I of the *cry4A* gene (762 bp, 35 kDa) was raised in mice. The effect of glucose on Cry4A expression in SK2 and SK9 is in accordance with the effect on mRNA observed earlier. The 130-kDa Cry4A protein was repressed by glucose in the wild-type strain but not in the $G_{49} \rightarrow A$ variant as expected (Fig. 5B). In addition, the amount of Cry4A synthesis in the variant strain was also higher than that in the wild-type cells.

To allow smooth operation of essential metabolic processes that share common transcription factors in the mother cell, a stringent control of *cry* genes seems imperative. Since σ subunits play a key role in temporal control of Cry4A synthesis as well as sporulation in *Bacillus* (16, 25, 34), maintenance of adequate σ^{35} and σ^{28} levels is crucial for the cell. A high transcription rate of *cry4A* is likely to titrate out the cellular σ subunit pool and may lead to disruption of the developmental process. Indeed suppression of sporulation was observed earlier in the *ptsH* knockout mutant (16) and also in a separate study during overexpression of the recombinant Cry proteins (1, 27).

In conclusion, we demonstrate a *cis*-acting regulatory sequence, the *cre* associated with the *cry4A* promoter, which performs a vital function for the evolutionary success of the cell. The importance of this regulatory element lies in maintaining normal cellular growth, development and synthesis of large amounts of toxin protein in *B. thuringiensis* subsp. *israelensis*. Considering the significance of *B. thuringiensis* as the most successful biopesticide, understanding toxin protein regulation is the key to maximizing protein yield for biotechnological applications.

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