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Loss of Catabolite Repression Function of HPr, the Phosphocarrier Protein of the Bacterial Phosphotransferase System, Affects Expression of the *cry4A* Toxin Gene in *Bacillus thuringiensis* subsp. *israelensis*

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HPr, the phosphocarrier protein of the bacterial phosphotransferase system, mediates catabolite repression of a number of operons in gram-positive bacteria. In order to participate in the regulatory process, HPr is activated by phosphorylation of a conserved serine-46 residue. To study the potential role of HPr in the regulation of Cry4A protoxin synthesis in *Bacillus thuringiensis* subsp. *israelensis*, we produced a catabolite repression-negative mutant by replacing the wild-type copy of the *ptsH* gene with a mutated copy in which the conserved serine residue of HPr was replaced with an alanine. HPr isolated from the mutant strain was not phosphorylated at Ser-45 by HPr kinase, but phosphorylation at His-14 was found to occur normally. The enzyme I and HPr kinase activities of the mutant were not affected. Analysis of the *B. thuringiensis* subsp. *israelensis* mutant harboring *ptsH*-S45A in the chromosome showed that *cry4A* expression was derepressed from the inhibitory effect of glucose. The mutant strain produced both *cry4A* and σ^{35} gene transcripts 4 h ahead of the parent strain, but there was no effect on σ^{28} synthesis. In wild-type *B. thuringiensis* subsp. *israelensis* cells, *cry4A* mRNA was observed from 12 h onwards, while in the mutant it appeared at 8 h and was produced for a longer period. The total amount of *cry4A* transcripts produced by the mutant was higher than by the parent strain. There was a 60 to 70% reduction in the sporulation efficiency of the mutant *B. thuringiensis* subsp. *israelensis* strain compared to the wild-type strain.

Biological control of dipteran pests in general and mosquitoes in particular has been a subject of primary importance for many years. *Bacillus thuringiensis* subsp. *israelensis* has been found to be the most effective microbial strain to date, possessing all the desirable properties of an ideal biocontrol agent. *B. thuringiensis* subsp. *israelensis* produces larvicidal crystal proteins in the stationary phase, concomitant with sporulation. The larvicidal activity of the parasporal crystals is attributed to the δ-endotoxin, composed of at least four major polypeptide species of about 27, 72, 128, and 135 kDa, which act synergistically in the manifestation of toxicity (27, 32). The high levels of toxin accumulation are controlled by a variety of mechanisms at the transcriptional, posttranscriptional, and posttranslational levels (2). The genes encoding different protein toxins are normally associated with large plasmids in *B. thuringiensis* subsp. *israelensis* (20, 26) and are named *cry4A*, *cry4B*, *cry11A*, and *cytA. cry4A* and *cry4B* code for the 135-kDa and 125-kDa protoxins, respectively, which are activated by the gut proteases in the alkaline conditions of the insect gut.

In sporulating cells of *B. thuringiensis*, the accumulation of large amounts of toxin proteins is achieved by expression from strong promoters associated with the gene. As in *Bacillus subtilis,* the developmental process is temporally and spatially regulated at the transcriptional level in *B. thuringiensis* by successive activation of different σ factors (6). In *B. thuringiensis*, the σ^{28} and σ^{35} subunits of RNA polymerase are considered functionally equivalent to $E\sigma^{K}$ and $E\sigma^{E}$, respectively, of *B. subtilis* and have been shown to direct toxin gene transcription during the sporulation phase (1). Most *cry4A* gene transcription occurs by the early sporulation-specific σ^{35} protein, from a strong *B. thuringiensis* I promoter (35, 36). Later, in the mid-sporulation phase, σ^{28} takes over and directs transcription from the *B. thuringiensis* II promoter (28, 37), resulting in sustained toxin synthesis over a long period of time.

In gram-positive bacteria, catabolite repression of many catabolic operons has been shown to involve a small phosphoprotein, HPr, which is considered a key component of the signal transduction cascade representing catabolite repression. The data emerging from a number of studies (10, 12, 13, 31) point to the metabolite-activated phosphorylation of the phosphocarrier protein HPr on the conserved serine-46 as a critical step. Activation of HPr occurs under conditions favoring growth and high glycolytic activity. Once activated, the phosphorylated HPr binds to the catabolite control protein (CcpA), forming a complex with strong DNA binding affinity (12, 16). The binding of the phosphorylated serine-HPr-CcpA complex to the 14-bp *cre* sequence (33) associated with the target DNA leads to its transcriptional modulation (15).

Earlier we reported the effect of glucose and inorganic phosphate on Cry4A toxin synthesis (4). The modulation of toxin synthesis by both glucose and inorganic phosphate was found to occur at the mRNA level, suggesting the potential involvement of catabolite repression in the regulation. It was also observed that HPr phosphorylation and dephosphorylation in

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TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Relevant characteristics	Reference
Plasmids		
pSR2	pGEM-T easy containing a 293-bp PCR-amplified *ptsH*	18
pSR15	pGEM-T easy containing 293-bp *ptsH* S45A	This work
pSR16	pGEM-T easy containing 293-bp *ptsH* S45A and kanamycin resistance cassette	This work
pSR18	pGEM-T easy containing 700-bp *B. thuringiensis* subsp. *israelensis* σ^{33}	This work
pSR19	pGEM-T easy containing 700-bp *B. thuringiensis* subsp. *israelensis* σ^{28}	This work
pDG783	pSB118 containing kanamycin cassette at *Hin*dIII site	14
Strains		
SR2	*E. coli* DH5α cells harboring 293-bp *ptsH* gene of *B. thuringiensis* subsp. *israelensis* in pGEM-T easy	18
SR15	*E. coli* DH5α cells containing 293-bp *ptsH* S45A gene in pGEM-T easy	This work
SR16	*E. coli* RR1 cells containing 293-bp *ptsH* S45A and kanamycin cassette in pGEM-T easy	This work
SR17	*B. thuringiensis* subsp. *israelensis* with *ptsH* S45A integrated downstream of wild-type *ptsH* promoter in chromosome	This work
E. coli RR1	*hsdS20* (r^-_B m^+_B) *supE44 ara-14 proA2 rspL20*(Str^r) *lacY1 galK2 xyl-5 mtl-1 supE44*	Promega
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M1515* Tn*10*(Tet^r)]	Stratagene

B. thuringiensis subsp. *israelensis* correlated with the glucose-mediated repression of Cry4A toxin synthesis (5).

In the present work, we investigated the role of HPr during glucose repression of Cry4A toxin synthesis in *B. thuringiensis* subsp. *israelensis* at the genetic level by inactivating the regulatory function of the *ptsH* gene, encoding the protein HPr. Since phosphorylation of the conserved serine-45 residue of HPr is primarily responsible for its regulatory catabolite repression function in *B. thuringiensis* subsp. *israelensis* (18), we first created a point mutation in the *ptsH* gene of *B. thuringiensis* subsp. *israelensis* by replacing the Ser-45 residue with alanine. The mutated copy of the gene was integrated into the bacterial chromosome to replace the wild-type copy by homologous recombination. We present data analyzing the effect of glucose on the synthesis of Cry4A toxin in the mutant strain.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *B. thuringiensis* subsp. *israelensis* strain HD522 (American Type Culture Collection) was grown in Luria-Bertani medium, G-medium (34), or Hogg-Jago medium (25) at 29°C as specified for different experiments. For isolation of DNA, the cultures were grown overnight in Luria-Bertani medium. The cell pellet was processed for chromosomal DNA preparation as described by Ausubel et al. (3). For estimating toxin-specific mRNA and toxin protein synthesis, the cultures were grown in G salts as described before (4). Briefly, the cells were grown with shaking for 14 to 15 h, washed once, and resuspended in starvation medium (RM) containing 50 mM Tris-HCl buffer (pH 7.5), 0.2% $(NH_4)_2SO_4$, 0.008% $CaCl_2$, 0.025% $FeSO_4 \cdot 5H_2O$, 0.05% $CuSO_4 \cdot 5H_2O$, 0.05% $ZnSO_4 \cdot 7H_2O$, 0.5% $MnSO_4 \cdot H_2O$, and 2.0% $MgSO_4$.

DNA manipulations. The *ptsH* gene from *B. thuringiensis* subsp. *israelensis* was cloned as described earlier (18). The point mutation in the *ptsH* gene was incorporated by using the construct pSR2 (Table 1) (18), which contained the wild-type copy of the *ptsH* gene. Site-directed mutagenesis was performed to replace the serine-45 residue with an alanine with the QuikChange mutagenesis kit (Stratagene). The oligonucleotide primers were 5′-GTTAACTTAAAAGCA ATCATGGGCGTAATG-3′ and 5′-CATTACGCCCATGATTGCTTTTAAGT TAAC-3′. Some of the transformed colonies were sequenced to confirm the mutated residue, and one clone, SR15, was selected for further studies.

pSR15 DNA containing the S45A mutation in the *ptsH* gene was digested with *Hin*dIII (the site was created at the C-terminal part of the gene during cloning) for ligating a 1.5-kb kanamycin resistance cassette obtained by *Hin*dIII digestion of pDG783 (14). The ligation mix was transformed in *Escherichia coli* RR1 cells, and colonies were selected on plates containing ampicillin plus kanamycin. Plasmid DNA from clone SR16 was prepared by the alkaline lysis method and electroporated into *B. thuringiensis* subsp. *israelensis* cells.

Electroporation of *B. thuringiensis* subsp. *israelensis*. *B. thuringiensis* subsp. *israelensis* cells were grown overnight in Hogg-Jago medium, subcultured, and incubated until the A_{600} reached 0.5. Sterile glycine was added to the growing cells at a final concentration of 8 to 12%, and the cells were grown for another hour. The cells were centrifuged and washed thrice in cold sterile EPBS (5 mM phosphate buffer [pH 8.0], 10% glycerol, 40% sorbitol), and the final pellet was resuspended in 1.0 ml of EPBS buffer. Then 200 μl of competent cells was electroporated with 1 to 5 μg of pSR16 DNA at 2 kV, 400 Ω, and 25 μF capacitance. Cold Hogg-Jago medium (800 μl) was added immediately, and the cells were incubated with shaking at 29°C for 2 h. The transformants were selected on Luria-Bertani plates containing 5 μg of kanamycin/ml.

Screening of transformants. The recombinants were obtained by a single crossover event which inserted the mutated copy of the *ptsH* gene together with the kanamycin cassette and the vector DNA between the wild-type *ptsH* gene and its promoter. Genomic DNA from several kanamycin-resistant colonies was digested separately with *Pst*I and *Hin*dIII. Southern hybridization was performed after separation on an agarose gel. The *Hin*dIII-digested DNA was probed with the 1.5-kb kanamycin gene, and the *Pst*I-digested DNA was hybridized with a 300-bp *ptsH* probe. One clone, SR17, showing a positive reaction with both the kanamycin gene and the *ptsH* probe was selected for further studies.

To identify the point mutation in the HPr of strain SR17, it was grown overnight in Luria-Bertani medium containing 0.5% glucose. The cells were washed and resuspended in 5 mM Tris-HCl (pH 7.5) and lysed by sonication. The cell homogenate was filtered through Centricon-30, and the filtrate was dialyzed extensively against the above buffer and concentrated. The concentrated proteins containing predominantly HPr were subjected to [γ-^{32}P]ATP- and FBP-mediated phosphorylation at Ser-45 by HPr kinase and to enzyme I (EI) and phosphoenolpyruvate (PEP)-activated phosphorylation at His-14 as described before (18). For analyzing the polar effects of insertion of *ptsH* on the *ptsI* gene, EI was prepared from SR17 cells and assayed as described earlier (18).

Growth and sporulation. The growth pattern of the mutant strain was compared with that of the wild-type *B. thuringiensis* subsp. *israelensis* strain in Luria-Bertani medium as well as in the defined G-medium by subculturing an overnight culture in the appropriate medium. The optical density was measured at 600 nm. For determining the sporulation efficiency of the two strains, the cultures were grown in G-medium containing 0.2% glucose. Samples were removed at the designated time, serially diluted in sterile phosphate-buffered saline and subjected to heat treatment at 80°C for 15 min. Unheated and heated samples were plated in duplicate to determine the total viable and heat-resistant cell counts. The results are representative of three separate experiments.

Total RNA extraction. Strain SR17 was grown in G-medium containing 0.2% glucose. Cells were centrifuged at 4°C after 14 to 15 h of growth and quickly resuspended in RM, which was supplemented or not with 0.5 or 1% glucose. Cultures (15 ml) were removed at different time intervals, centrifuged at 4°C, and frozen at −20°C until further use.

In another set of experiments, both the wild-type and mutant *B. thuringiensis* subsp. *israelensis* strains were grown in G-medium containing 0.2% glucose, and samples were removed from 6 h onwards. Total RNA was prepared to study the kinetics of synthesis of *cry4A*, σ^{35}, and σ^{28} gene-specific messages in the two strains by probing with specific DNA probes. Total RNA was prepared from the cells with the RNeasy kit (Qiagen). The concentrations of RNA were calculated spectrophotometrically, and aliquots containing 2 μg of RNA were loaded. The blot was subsequently probed with a 1.7-kb DNA fragment containing the promoter and the N terminus of the *cry4A* gene (obtained from a cloned 3.9-kb *B. thuringiensis* subsp. *israelensis* fragment containing the *cry4A* gene with its pro-

moter) and the entire σ^{35} and σ^{28} gene sequences and exposed for autoradiography.

Cloning of σ^{35} and σ^{28} genes of *B. thuringiensis* subsp. *israelensis*. Based on the sequences of the σ^{35} and σ^{28} genes of *Bacillus thuringiensis* subsp. *kurstaki* HD1 (1), homologous sequences from *B. thuringiensis* subsp. *israelensis* were amplified by PCR and cloned, producing plasmids pSR18 and pSR19, respectively (GenBank accession numbers AY083614 and AY083615, respectively).

Uptake of 2-[^{14}C]deoxyglucose. One milliliter of culture (A_{600}, 0.8) of both the wild-type and the mutant strain was centrifuged and washed with 50 mM phosphate buffer (pH 7.5) at 4°C and resuspended in the same buffer. 2-[^{14}C]deoxyglucose was added to a final concentration of 2 mM (45 μCi/μmol) in a total volume of 500 μl. The cells were incubated at 29°C, and 25-μl samples were removed at different intervals up to 2 min and passed through a 0.2-μm filter. The filters were washed three times with 1 ml of 50 mM phosphate buffer and air dried. The filters were put in Aquasol, and the radioactivity associated with the cells was counted in a liquid scintillation counter.

Effect of glucose on Cry4A toxin synthesis. *B. thuringiensis* subsp. *israelensis* and SR17 cells were grown in G-medium overnight at 29°C and resuspended in RM after 15 h of growth. Glucose was added to a final concentration of 0.5 and 1% to the RM. Two-milliliter samples were removed at 12 and 14 h after resuspension, and aliquots containing equal amounts of proteins (22) were solubilized in loading dye and resolved by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were visualized by Coomassie blue staining. For Western blotting, antibodies were raised against Cry4A protoxin isolated from the recombinant *B. thuringiensis* subsp. *israelensis* strain 4Q2-81(pHT606) containing the *cry4A* protoxin gene only (8).

RESULTS

Preparation of HPr-S45A mutant. The PCR-amplified nicked circular product containing the S45A mutation in the *ptsH* gene was obtained at 60°C. It was transformed into *E. coli* to produce clone SR15, which contained the desired mutation.

The plasmid DNA obtained from clone SR16, a derivative of pSR15 (Fig. 1), contained the 1.5-kb kanamycin cassette at the *Hin*dIII site at the 3′ end of the *ptsH* gene. Electroporation of the pSR16 DNA into *B. thuringiensis* subsp. *israelensis* occurred at a rather low frequency (5×10^{-7}). In the Southern blot, a 6.5-kb fragment in the *Pst*I-digested genomic DNA of SR17 reacted with the 300-bp *ptsH* probe, which was absent in the wild-type strain (data not presented). Hybridization of the *Hin*dIII-digested genomic DNA with the kanamycin gene sequence yielded two bands at 1.5 and 4.5 kb, and both the bands were absent in the wild-type *B. thuringiensis* subsp. *israelensis* DNA (data not shown), indicating that the kanamycin cassette had integrated in the *B. thuringiensis* subsp. *israelensis* chromosome at the designated location.

Identification of point mutation. In contrast to the wild-type protein, no phosphorylation was observed when the same amount of HPr prepared from strain SR17 was subjected to phosphorylation at Ser-45 by HPr kinase and [γ-^{32}P]ATP (Fig. 2A, lanes 1 and 2). As expected, there was no effect on the EI-mediated phosphorylation at His-14 (Fig. 2B). The above result demonstrated that in the mutant, the wild-type copy of the *ptsH* gene is totally silent, and the HPr produced by the latter is the altered HPr-S45A only. The mutant strain was able to produce HPr kinase normally, as shown in Fig. 2A, lanes 3 and 4, in which HPr kinases prepared from the wild-type strain and the mutant strain were used to phosphorylate recombinant *B. thuringiensis* subsp. *israelensis* HPr at Ser-45. Furthermore, partially purified cell homogenate from strain SR17, used as a source of EI, was able to transfer the phosphoryl group to recombinant *B. thuringiensis* subsp. *israelensis* HPr as efficiently as the wild-type strain (data not shown). This indicated that there was no polar effect on the expression of the *ptsI* gene, and the second copy of the *ptsH* gene is not expressed.

Growth, sporulation, and 2-[^{14}C]deoxyglucose uptake. The growth kinetics of the two strains in G-medium and in Luria-Bertani medium were identical (Fig. 3A and B). The cultures grew exponentially up to 6 to 7 h and entered the stationary phase after 8 h. Sporulation efficiency was determined after 20 h, and the time of appearance of heat-resistant spores varied in different experiments and occurred between 18 and 24 h. The mutant strain showed a 60 to 70% reduction in the formation of heat-resistant spores. The sporulation efficiency in the latter was 20 to 30%, compared to more than 80% observed in the wild-type strain after 24 h (Fig. 3C). The rate of glucose uptake by both strains was similar, as shown in Fig. 3D. The rate calculated in the initial 30 s was almost the same in both cases.

Effect of glucose on *cry4A* gene expression. *cry4A*-specific mRNA levels were reduced in the presence of glucose in the wild-type but not in the mutant strain (Fig. 4A and B). The concentration of *cry4A* transcripts was considerably higher at the time of resuspension (0 h) in the mutant strain than in the wild-type (Fig. 4A and B, lane 1). *cry4A* mRNA could be detected till 5 h after resuspension in both cases. In the parent strain, the transcript levels were drastically reduced within 15 min of addition of glucose, but there was no effect in the mutant cells (Fig. 4A, lane 2).

Cry4A protein synthesis was monitored in the cultures after resuspension in starvation medium with or without glucose. Similar to its effect on the mRNA levels, a corresponding effect could be observed on the synthesis of Cry4A protoxin. When the cells were collected 12 to 14 h after resuspension and total proteins were subjected to SDS-PAGE, the response of the two strains was markedly different in the presence of glucose. Glucose at 0.5 and 1% (Fig. 5, lanes 2 and 3) inhibited protoxin synthesis in the wild-type strain, but the mutant was totally insensitive to glucose (Fig. 5, lanes 4, 5, and 6). The location of the Cry4A protein was determined by blotting the separated proteins with Cry4A-specific antibodies (data not shown). Other toxin or nontoxin proteins apparently remained unaffected.

Kinetics of *cry4A*, σ^{35}, and σ^{28} mRNA synthesis. The two strains differed in the time of appearance as well as the level of *cry4A* mRNA (Fig. 6A). In the wild-type *B. thuringiensis* subsp. *israelensis*, the mRNA could be detected from 12 to 14 h of growth, and its levels were lower than that obtained in the mutant strain, in which the transcripts could be detected from 8 h onwards (Fig. 6A). In both strains, the *cry4A* transcripts persisted beyond the 14th hour of growth.

Since σ^{35} is the major sigma factor involved in transcription of the *cry4A* gene during sporulation, its profile was determined to see if the loss in activity of the *ptsH* gene had any effect on the levels of σ^{35} protein (Fig. 6B). σ^{35}-specific mRNA was detectable between 12 and 14 h in the wild-type strain; the timing matched the appearance of *cry4A* mRNA (Fig. 6A and B). In the mutant strain, σ^{35} transcripts were visible much earlier, at 8 h, and remained until 12 h, coinciding with the synthesis of *cry4A* mRNA. There was no difference in the levels of the σ^{35} transcripts produced in the two strains (Fig. 6B). These results indicate that σ^{35} gene transcription is also regulated by a *ptsH*-mediated repression in *B. thuringiensis* subsp.

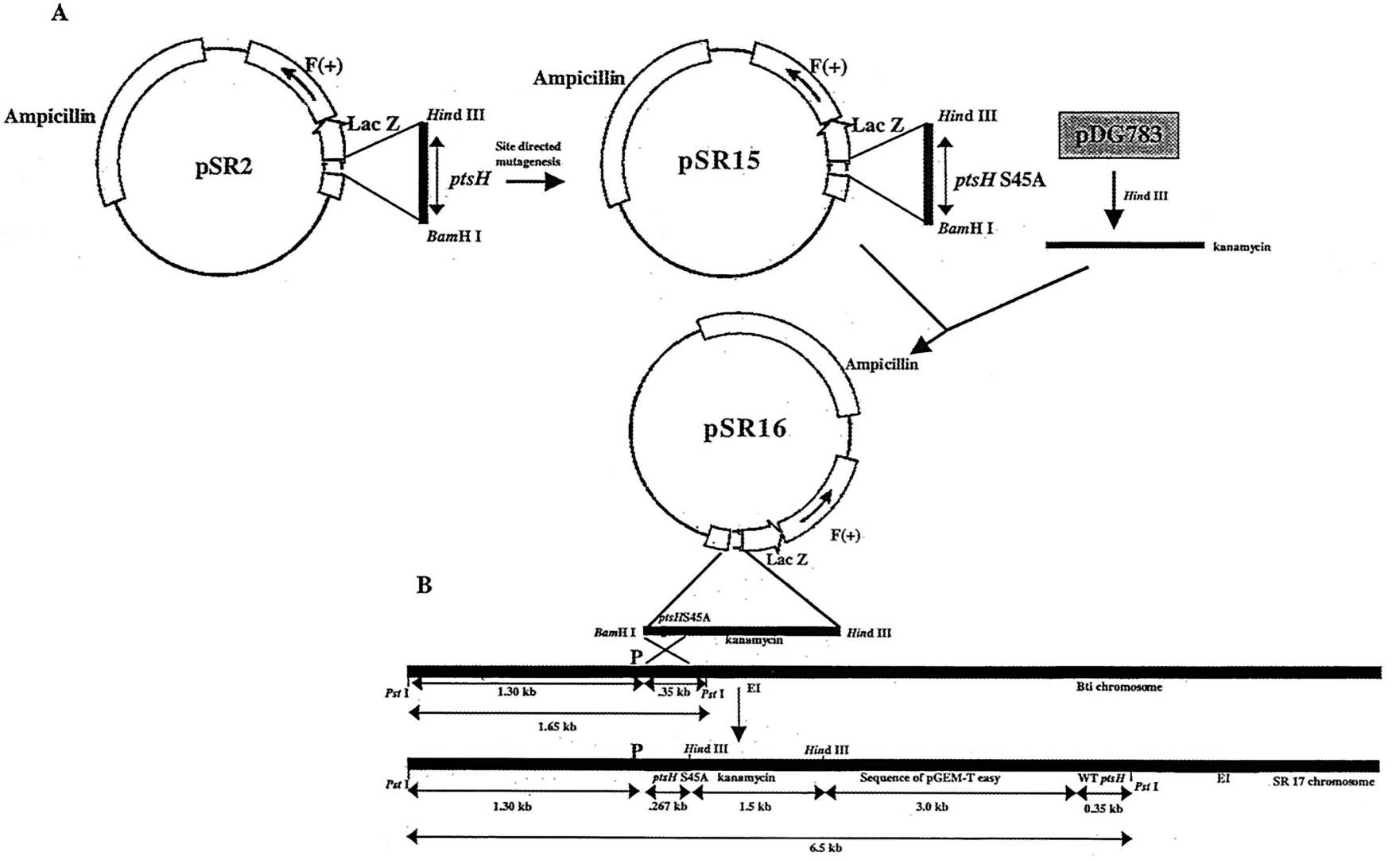

FIG. 1. Construction of *B. thuringiensis* subsp. *israelensis* mutant SR17, containing the *ptsH* S45A allele in the chromosome. (A) The wild-type promoterless *ptsH* gene in pSR2 was mutagenized by reverse PCR with primers containing the mutated residue. A kanamycin resistance cassette was incorporated at the *Hin*dIII site of pSR15, producing pSR16. (B) Homologous recombination of pSR16 by single crossing over at the *ptsH* locus resulted in integration of the *ptsH* S45A allele downstream of the wild-type *ptsH* promoter, rendering the wild-type (wt) *ptsH* gene nonfunctional. The size of the *Pst*I-digested fragment containing the wild-type *ptsH* gene increased from 1.65 to 6.5 kb due to duplication of the gene and addition of the vector DNA and kanamycin cassette between two *ptsH* copies.

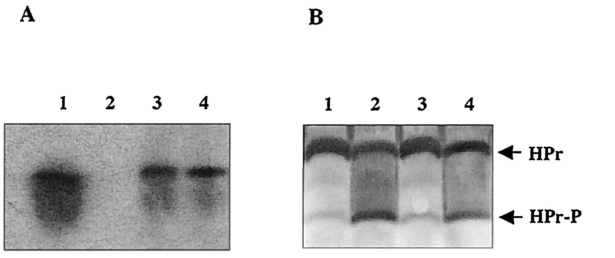

FIG. 2. Phosphorylation pattern of HPr from wild-type *B. thuringiensis* subsp. *israelensis* and *ptsH* S45A mutant strain SR17. (A) Autoradiogram showing phosphorylation of HPr by HPr kinase and [γ-^{32}P]ATP at serine-45. Lane 1, HPr from *B. thuringiensis* subsp. *israelensis* phosphorylated by HPr kinase from *B. thuringiensis* subsp. *israelensis*; lane 2, HPr from SR17 phosphorylated by HPr kinase from SR17; lanes 3 and 4, *B. thuringiensis* subsp. *israelensis* recombinant HPr phosphorylated by HPr kinase from *B. thuringiensis* subsp. *israelensis* and SR17, respectively. (B) Western blot of nondenaturing polyacrylamide gel, showing phosphorylation of HPr by PEP and recombinant EI from *B. subtilis* at histidine-14. Lane 1, HPr from *B. thuringiensis* subsp. *israelensis*; lane 2, HPr from *B. thuringiensis* subsp. *israelensis* phosphorylated by EI and PEP; lane 3, HPr from SR17; lane 4, HPr from SR17 phosphorylated by EI and PEP.

israelensis. The time of appearance and concentration of σ^{28} were not affected in the *ptsH* mutant (Fig. 6C), indicating the insensitivity of σ^{28} to *ptsH*-mediated regulation.

DISCUSSION

The results described in this study demonstrate that phosphorylation of HPr at serine-45 mediates glucose repression of Cry4A protoxin synthesis in *B. thuringiensis* subsp. *israelensis*. Glucose catabolite repression is considered a global regulatory mechanism, controlling the activity of many catabolic operons (9, 12, 13, 31) in response to carbon source availability in bacteria. We created a catabolite repression-negative *ptsH* mutant of *B. thuringiensis* subsp. *israelensis*, in which an increase in *cry4A* gene transcription has occurred at the expense of the developmental process. Bryan et al. described regulation of the σ^{E}-dependent *mmg* operon by glucose catabolite repression in *B. subtilis* (7), providing the rationale for such a regulation in maintaining the optimum metabolic state of the mother cell during endospore development. Another Eσ^{E}-driven operon,

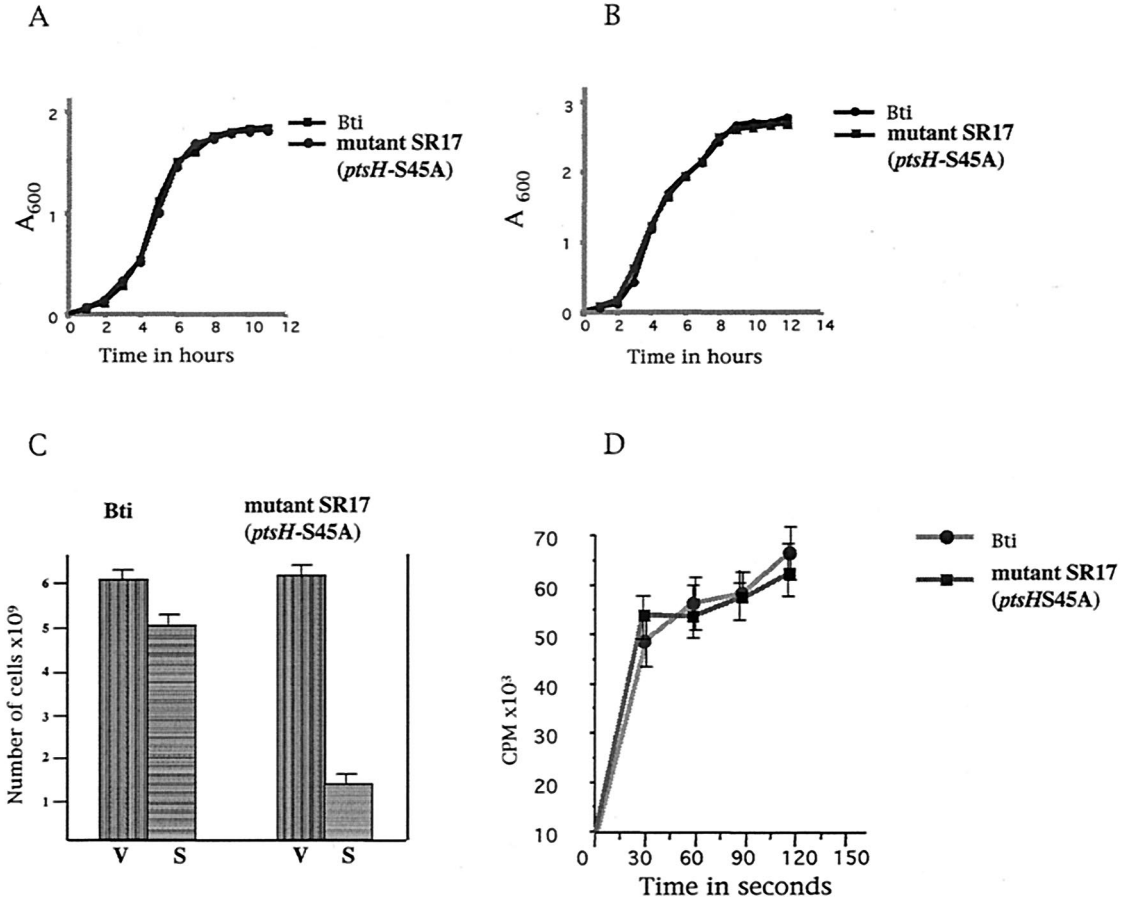

FIG. 3. Growth and sporulation characteristics of *ptsH* mutant strain SR17. (A) Growth patterns of wild-type *B. thuringiensis* subsp. *israelensis* (Bti) and *ptsH* mutant strain SR17 in G-medium. (B) Growth patterns of wild-type *B. thuringiensis* subsp. *israelensis* and *ptsH* mutant strain SR17 in Luria-Bertani medium. (C) Comparison of sporulation efficiencies of the two strains in G-medium. The formation of heat-resistant cells was monitored after growing the strains in G-medium for 20 h. V, total viable cell counts; S, heat-resistant cell counts. (D) Uptake of 2-[^{14}C]deoxyglucose by the wild-type and mutant strains. Cells in the mid-exponential phase were resuspended in the presence of 2-[^{14}C]deoxyglucose, and the incorporation of radioactivity by the cells was monitored by liquid scintillation counting. The mean values of three independent experiments have been plotted, and the standard deviations have been plotted as error bars.

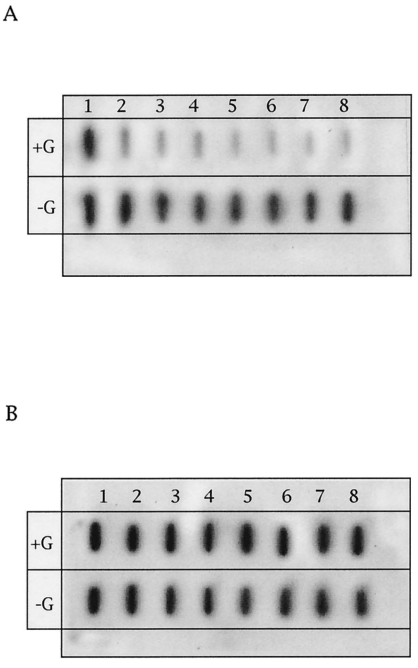

FIG. 4. Effect of glucose on *cry4A*-specific mRNA in *B. thuringiensis* subsp. *israelensis* and the *ptsH* S45A mutant. Both strains were grown in G-medium for 15 h and resuspended in RM with or without 0.5% glucose. Then 15-ml samples were removed at different times, and total RNA was prepared and probed with a 1.7-kb *cry4A*-specific DNA probe. (A) Total RNA from *B. thuringiensis* subsp. *israelensis*. (B) Total RNA from *ptsH* mutant SR17 (*ptsH* S45A). Lanes 1 to 8, samples collected at 0 h, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h, and 5 h, respectively.

the glycogen operon, which is repressed by glucose in sporulating cells of *B. subtilis*, also supports the need for metabolic control of the stationary-phase phenomenon (19).

In the light of the remarkable conservation of sigma subunits between *B. thuringiensis* and *B. subtilis*, the temporal regulation of sporulation in *B. thuringiensis* through the cascade of sigma factors is believed to be closely similar to that of *B. subtilis* (1, 21, 23, 24). Homologs of several important transcription factors described in *B. subtilis* have been identified in *B. thuringiensis* also (1). In *B. thuringiensis* subsp. *israelensis*, the total Cry4A protein produced is the sum of three different promoter activities. In the stationary phase, σ^H-containing RNA polymerase initiates low-level transcription (28), followed by σ^{35} in the mid-sporulation phase (35), and finally σ^{28}-directed transcription occurs from a weak promoter in the late sporulation stages (37). The expression of the $E\sigma^E$ precursor in *B. subtilis* starts just before septation by σ^A-containing RNA polymerase in conjunction with Spo0A (11, 17, 29), a protein belonging to the family of global response regulator proteins (30).

In the event of a similar mechanism operating in *B. thuringiensis* subsp. *israelensis*, an additional control through *ptsH* on either of these genes active in the vegetative phase would serve as a switch linked to the metabolic state of the cell. In the wild-type *B. thuringiensis* subsp. *israelensis*, *cry4A* mRNA was detected between 12 and 14 h, with the simultaneous induction of σ^{35} mRNA. Very low transcription of *cry4A* occurred before that, indicating the predominance of the σ^{35}-directed synthesis of *cry4A* transcription in that period, while σ^H promoter activity remained very low. Significantly, in the *ptsH* mutant, σ^{35} transcripts appeared 4 h earlier than in the wild type, at 8 h of growth, with concomitant synthesis of *cry4A* mRNA.

Derepression of both genes in the *ptsH* mutant strongly

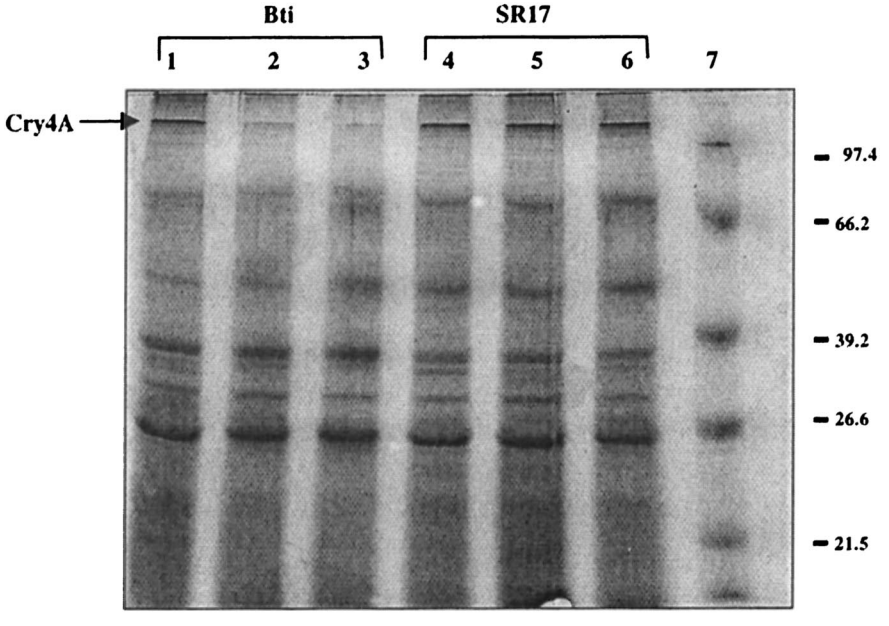

FIG. 5. Protein profiles of wild-type *B. thuringiensis* subsp. *israelensis* (Bti) and the *ptsH* S45A mutant, showing the effect of glucose on Cry4A synthesis. Both strains were grown in G-medium for 15 h and resuspended in RM containing 0, 0.5, or 1% glucose. Aliquots of cells were removed 12 h after resuspension, and the proteins were solubilized in SDS sample buffer and resolved on 10% polyacrylamide gels. Lanes 1 and 4, no glucose; lanes 2 and 5, 0.5% glucose; lanes 3 and 6, 1% glucose; lane 7, molecular size markers, indicated at the right in kilodaltons.

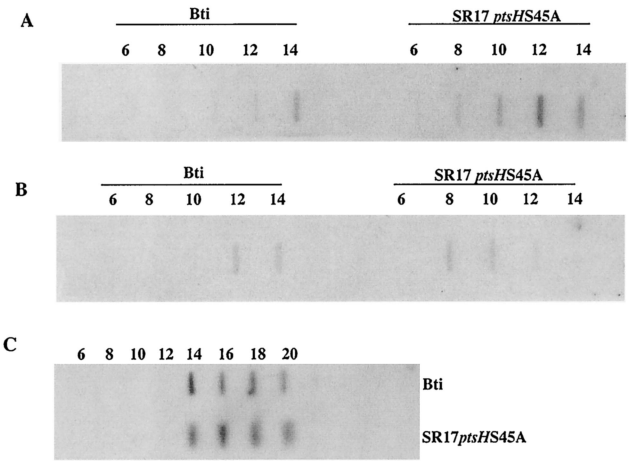

FIG. 6. mRNA profiles of σ^{35}, σ^{28}, and *cry4A* in *B. thuringiensis* subsp. *israelensis* (Bti) and SR17 (*ptsH* S45A) mutant during growth. Both strains were grown in G-medium, and cell samples were removed at different times. Total RNA was prepared from these cells and probed with *cry4A*-, σ^{35}-, or σ^{28}-specific probes to determine the transcript level. (A) Profile of *cry4A*-specific mRNA. (B) Profile of σ^{35}-specific mRNA. (C) Profile of σ^{28}-specific mRNA. Slots 1 to 8, samples removed at 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, and 20 h, respectively.

supports the key role of *ptsH*-mediated regulation of Cry4A toxin synthesis. The regulation is apparently exerted through primary modulation of the σ^{35} gene or the proteins responsible for its transcription, i.e., σ^{A} or Spo0A (20, 28). It is necessary to mention here that two potential *cre* sequences at −488 and +63 nucleotides were found in the upstream and coding regions of the σ^{35} gene of *B. thuringiensis* subsp. *kurstaki* (1). In the later periods (12 to 14 h), the *cry4A* gene is apparently transcribed by both σ^{35} and σ^{28} in the *ptsH* mutant, as shown by the σ^{28} profile.

As a consequence of early induction of Eσ^{35} in the mutant strain, an enhancement in sporulation efficiency parallel to toxin synthesis was expected. However, contrary to our expectations, sporulation was inhibited in the mutant in comparison to the wild-type strain. Derepression of negative regulators of sporulation from *ptsH*-mediated catabolite repression in the catabolite repression-negative strain could be one of the reasons for this. Alternatively, the untimely induction of Eσ^{35} protein in cells metabolically not ready to start the developmental process could be another reason for aborted spore development, and this is supported by our kinetics data on the induction of *cry4A* and the sigma factor genes. The third reason could be titration of Eσ^{35} by the high-throughput *cry* gene promoters in the absence of the appropriate sporulation machinery, leading to increased transcription of the *cry4A* gene at the expense of the developmental process. The reduced sporulation efficiency of the *ptsH* mutant observed in this study reinforces the basic argument that catabolite repression of the toxin gene in the early stages is necessary for providing adequate levels of the sigma factors to the developmental machinery.

Interestingly, the concentration of *cry4A* transcripts remained high till 14 h of growth in the mutant, when transcription occurred mainly by a σ^{28}-controlled promoter (Fig. 6A and C). However, our resuspension experiments with cells of similar age (15 h old) showed that glucose repressed *cry4A* transcription in the wild-type *B. thuringiensis* subsp. *israelensis* cells but not in the *ptsH* mutant. Considering the fact that the *ptsH* mutation had no effect on the σ^{28} profile, the insensitivity of the *ptsH* mutant to glucose in this period also suggests an additional *ptsH*-mediated control on the toxin gene, which is probably required for finer regulation of toxin synthesis.

TABLE 2. Putative *cre* sequences present in *cry4A*

cre (position)	Sequence
Consensus	TG(A/T)∗A∗CG∗T∗(T/A)CA
−6 to −19	5′-AA A TTACGAAT A CT-3′
+246 to +259	5′-TG G TTTCGGGT T CA-3′
+591 to +604	5′-TG A TTGCGATT A CT-3′
+3504 to +3517	5′-TT A TATCGAAA G CA-3′

We have identified several putative *cre* sequences (33) in the upstream region and also within the coding sequence of the *cry4A* gene (Table 2). Studies are in progress to verify their role in regulation. The presence of a functional *cre* sequence downstream of the promoters or within the open reading frame could be expected to result in regulation of *cry4A* gene transcription dependent on the concentration of carbon metabolites.

In conclusion, the analysis of *cry4A* gene transcription in a *ptsH* mutant of *B. thuringiensis* subsp. *israelensis* demonstrates that protoxin synthesis is indeed controlled by *ptsH*-mediated glucose catabolite repression. The primary target of the regulatory process could not be ascertained in this study. Significantly, the *ptsH* mutant strain of *B. thuringiensis* subsp. *israelensis* has the potential to produce larger amounts of toxin than the wild-type strain over a longer period of time. This study demonstrates for the first time the key role of HPr in the modulation of Cry4A toxin expression in *B. thuringiensis* subsp. *israelensis*.

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REFERENCES

1. **Adams, L. F., K. L. Brown, and H. R. Whiteley.** 1991. Molecular cloning and characterization of two genes encoding sigma factors that direct transcription from a *Bacillus thuringiensis* crystal protein gene promoter. J. Bacteriol. **173:**3846–3854.
2. **Agaisse, H., and D. Lereclus.** 1995. How does *Bacillus thuringiensis* produce so much insecticidal crystal protein? J. Bacteriol. **177:**6027–6032.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology, vol. I and II. John Wiley and Sons, New York, N.Y.
4. **Banerjee-Bhatnagar, N.** 1998. Modulation of CryIVA toxin protein expression by glucose in *Bacillus thuringiensis israelensis*. Biochem. Biophys. Res. Commun. **252:**402–406.
5. **Banerjee-Bhatnagar, N.** 1999. Inorganic phosphate regulates CryIVA protoxin expression in *Bacillus thuringiensis israelensis*. Biochem. Biophys. Res. Commun. **262:**359–364.
6. **Baum, J. A., and T. Malvar.** 1995. Regulation of insecticidal crystal protein production in *Bacillus thuringiensis*. Mol. Microbiol. **18:**1–12.
7. **Bryan, E. M., B. W. Beall, and C. P. Moran, Jr.** 1996. A σ^{E}-dependent operon subject to catabolite repression during sporulation in *Bacillus subtilis*. J. Bacteriol. **178:**4778–4786.
8. **Delecluse, A., S. Poncet, A. Klier, and G. Rapoport.** 1993. Expression of *cry4A* and *cry4B* genes, independently or in combination, in a crystal-negative

strain of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **59:**3922–3927.

9. **Deutscher, J., J. Reizer, C. Fischer, A. Galinier, M. H. Saier, Jr., and M. Steinmetz.** 1994. Loss of protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolic genes of *Bacillus subtilis*. J. Bacteriol. **176:**3336–3344.
10. **Deutscher, J., E. Kuster, U. Bergstedt, V. Charrier, and W. Hillen.** 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. Mol. Microbiol. **15:** 1049–1053.
11. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
12. **Fujita, Y., Y. Miwa, A. Galinier, and J. Deutscher.** 1995. Specific recognition of the *Bacillus subtilis gnt cis*-acting catabolite-responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. Mol. Microbiol. **17:**953–960.
13. **Galinier, A., J. Deutscher, and I. Martin-Verstraete.** 1999. Phosphorylation of either Crh or HPr mediates binding of CcpA to the *Bacillus subtilis xyn cre* and catabolite repression of *xyn* operon. J. Mol. Biol. **286:**307–314.
14. **Guerout-Fleury, A.-M., K. Shazand, N. Frandsen, and P. Stragier.** 1995. Antibiotic resistance cassettes for *Bacillus subtilis*. Gene **167:**335–336.
15. **Hueck, C. J., and W. Hillen.** 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? Mol. Microbiol. **15:**395–401.
16. **Jones, E. B., V. Dossonnet, E. Kuster, W. Hillen, J. Deutscher, and R. E. Klevit.** 1997. Binding of the catabolite repressor protein CcpA to its DNA target is regulated phosphorylation of its corepressor HPr. J. Biol. Chem. **272:**26530–26535.
17. **Kenney, T. J., K. York, P. Youngman, and C. P. Moran, Jr.** 1989. Genetic evidence that RNA polymerase associated with σ^A factor uses a sporulation specific promoter in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **86:**9109–9113.
18. **Khan, S. R., J. Deutscher, R. A. Vishwakarma, V. Monedero, and N. Banerjee-Bhatnagar.** 2001. The *ptsH* gene from *Bacillus thuringiensis israelensis*. Characterization of a new phosphorylation site on the protein HPr. Eur. J. Biochem. **268:**521–530.
19. **Kiel, J. A. K. W., J. M. Boels, G. Beldman, and G. Venema.** 1994. Glycogen in *Bacillus subtilis*: molecular characterization of an operon encoding enzymes involved in glycogen biosynthesis and degradation. Mol. Microbiol.**11:** 203–211.
20. **Lereclus, D., A. Delecluse, and M.-M. Lecadet.** 1993. Diversity of *Bacillus thuringiensis* toxins and genes, p. 37–69. *In* P. Entwistle, J. S. Cory, M. J. Bailey, and S. Higgs (ed.), *Bacillus thuringiensis*, an environmental biopesticide: theory and practice. John Wiley and Sons, Ltd., Chichester, United Kingdom.
21. **Lereclus, D., H. Agaisse, M. Gominet, and J. Chaufaux.** 1995. Overproduction of encapsulated insecticidal crystal proteins in a *Bacillus thuringiensis* spo0A mutant. Bio/Technology **13:**67–71.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
23. **Malvar, T., C. Gawron-Burke, and J. A. Baum.** 1994. Overexpression of *Bacillus thuringiensis* HknA, a histidine protein kinase homolog, bypasses early Spo^- mutations that result in CryIIIA overproduction. J. Bacteriol. **176:**4742–4749.
24. **Malvar, T., and J. A. Baum.** 1994. Tn*5401* disruption of *spo0F* gene, identified by direct chromosomal sequencing, results in CryIIIA overproduction in *Bacillus thuringiensis*. J. Bacteriol. **176:**4750–4753.
25. **Marciset, O., and B. Mollet.** 1994. Multifactorial experimental designs for optimizing transformation: electroporation of *Streptococcus thermophilus*. Biotechnol. Bioeng. **43:**490–496.
26. **Margalith, Y., and E. Ben-Dov.** 2000. Biological control by *Bacillus thuringiensis* subsp. *israelensis*, p. 243–301. *In* Insect pest management: techniques for environmental protection. CRC Press, Boca Raton, Fla.
27. **Poncet, S., A. Delecluse, A. Klier, and G. Rappoport.** 1995. Evaluation of synergistic interactions among the CryIVA, CryIVB, and CryIVD toxic components of *B. thuringiensis* subsp. *israelensis* crystals. J. Invertebr. Pathol. **66:**131–135.
28. **Poncet, S., E. Dervyn, A. Klier, and G. Rappoport.** 1997. Spo0A represses transcription of the *cry* toxin genes in *Bacillus thuringiensis*. Microbiology **143:**2743–2751.
29. **Satola, S., P. A. Kirchman, and C. P. Moran, Jr.** 1991. Spo0A binds to a promoter used by σ^A RNA polymerase during sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88:**4533–4537.
30. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive response in bacteria. Microbiol. Rev. **53:**450–490.
31. **Stulke, J., I. Martin-Verstraete, V. E. Charrier, A. Klier, J. Deutscher, and G. Rappoport.** 1995. HPr protein of the phosphotransferase system links induction and catabolite repression of the *Bacillus subtilis* levanase operon. J. Bacteriol. **177:**6928–6936.
32. **Tabashnik, B. E.** 1992. Evaluation of synergism among *Bacillus thuringiensis* toxins. Appl. Environ. Microbiol. **58:**3343–3346.
33. **Weickert, M. J., and G. H. Chambliss.** 1990. Site directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**6238–6242.
34. **Wu, D., X. L. Cao, Y. Y. Bai, and A. I. Aronson.** 1991. Sequence of an operon containing a novel δ-endotoxin gene from *Bacillus thuringiensis*. FEMS Microbiol. Lett. **81:**32–36.
35. **Yoshisue, H., T. Fukada, K.-I. Yoshida, K. Sen, S.-I. Kurosawa, H. Sakai, and T. Komano.** 1993. Transcriptional regulation of *Bacillus thuringiensis* subsp. *israelensis* mosquito larvicidal crystal gene *cryIVA*. J. Bacteriol. **175:** 2750–2753.
36. **Yoshisue, H., K. Ihara, T. Nishimoto, H. Sakai, and T. Komano.** 1995. Expression of the genes for insecticidal proteins in *Bacillus thuringiensis cryIVA* not *cryIVB* is transcribed by RNA polymerase containing σ^H and that containing σ^E. FEMS Microbiol. Lett. **127:**65–72.
37. **Yoshisue, H., H. Sakai, K. Sen, M. Yamagiwa, and T. Komano.** 1997. Identification of a second transcriptional start site for the insecticidal protein gene *cryIVA* of *Bacillus thuringiensis* subsp. *israelensis*. Gene **185:**251–252.