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Expression of *Bacillus thuringiensis* δ-Endotoxin Genes during Vegetative Growth

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***Bacillus thuringiensis* δ-endotoxin (crystal protein) genes are normally expressed only during sporulation. It is possible to produce crystal protein during vegetative growth by placing *B. thuringiensis* crystal protein genes downstream of a strong vegetative promoter. By removing a possible transcriptional terminator of the tetracycline resistance gene of pBC16 and inserting a multiple cloning site, δ-endotoxin genes can be cloned downstream from the tetracycline resistance gene promoter. This construct allows for readthrough transcription from the strong vegetative promoter. Crystal protein is then produced during vegetative growth as well as during sporulation in both *B. thuringiensis* and *Bacillus megaterium*. This construct also allows for production of δ-endotoxin in *B. thuringiensis* strains that do not normally produce δ-endotoxin because of a defect in sporulation.**

Bacillus thuringiensis produces crystalline δ-endotoxins that are insecticidal. The crystals are ingested by the insect and cause cell lysis in the midgut and, eventually, the death of the insect (15). Most *B. thuringiensis* strains produce several different insecticidal δ-endotoxin proteins, and many strains produce a different complement of crystal proteins (11, 25). The δ-endotoxins are specific in their lethal activity against different insect pests and are not harmful to mammals, birds, or beneficial insects. For this reason, *B. thuringiensis* is being exploited as an alternative to chemical pesticides.

The production of δ-endotoxins is developmentally regulated. During stationary phase, the cells produce a spore and a parasporal inclusion body (2). Upon sporangial lysis, the crystal protein and the spore are released. δ-Endotoxin is not produced under conditions that do not permit sporulation or in certain sporulation mutants (19). The regulation of δ-endotoxin synthesis may share common features with the regulation of sporulation genes in *Bacillus subtilis*. In *B. subtilis*, the expression of sporulation genes is regulated, in part, by sporulation-specific sigma factors (14). A sporulation-specific sigma factor has recently been described in *B. thuringiensis*, and RNA polymerase in association with this sigma factor has been shown to transcribe several δ-endotoxin genes in vitro (4). Insecticidal δ-endotoxin genes have been cloned on high-copy-number vectors and introduced into *B. subtilis*, *Bacillus megaterium*, and *B. thuringiensis*. Like the native crystal gene, the cloned genes have been found to be developmentally regulated (5, 10, 27; A. Macaluso and A.-M. Mettus, unpublished results), though vegetative expression of certain cloned δ-endotoxin genes in *B. subtilis* and *B. megaterium* has been reported (22).

This work describes a novel vector enabling the expression of δ-endotoxin genes in *B. thuringiensis* and *B. megaterium* during vegetative growth. The expression of these genes is controlled by the promoter of the tetracycline resistance gene from the *Bacillus cereus* plasmid pBC16. The expression vector reported here alleviates the dependence of crystal production on sporulation or growth conditions that induce sporulation.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains used in this work are described in Table 1. *B. thuringiensis* HD73-26 is a crystal-negative, cured derivative of HD73 carrying only the 4.9-megadalton plasmid (6).

Plasmids. Plasmids used in this work are described in Table 2 and shown in Fig. 1. The nomenclature for δ-endotoxin genes used in this work is as defined by Hofte and Whiteley (8). Plasmid pBC16, encoding tetracycline resistance, was isolated from *Bacillus cereus* (3). Plasmid pEG19 contains, in pBR322, the *cryIA(c)* gene [identified as a 6.6-kilobase (kb) *Hin*dIII fragment that hybridized to a *cryIA* gene probe, with a restriction map consistent with that of *cryIA(c)*] on a 7.4-kb *Sph*I-*Nru*I fragment of DNA from *B. thuringiensis* HD263 (M. A. Von Tersch and H. Loidl Robbins, unpublished results). Plasmid pEG23 contains the *B. thuringiensis*-derived sequences from pEG19 inserted into the pBC16 derivative pNN101 (18). Plasmid pEG201 was constructed by the insertion into pBR322 of a 5.2-kb *Hin*dIII fragment of DNA from *B. thuringiensis* HD263 encoding *cryIIA* (5). Plasmid pEG204 was derived from pEG201 by the insertion of pBC16 at the unique *Sph*I site. Plasmids were constructed in this work in *Escherichia coli* RR1 or DH5α-competent cells from Bethesda Research Laboratories, Inc., in *E. coli* GM2163 (17) or in *B. cereus* BC569-6 (6).

Construction of the *cryIIA* plasmids pEG421 and pEG424. The 3.1-kb *Eco*RI fragment of pBC16 containing the origin of replication and the tetracycline resistance gene was ligated to the 8.5-kb *Eco*RI fragment of pEG201 containing the *cryIIA* gene, resulting in pEG421 and pEG424 (Fig. 1). The direction of transcription of *cryIIA* in pEG421 is opposite to that of *tet*, while in pEG424 it is in the same direction as that in the *tet* gene.

Construction of the *tet* expression vector pEG434. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer (model 38B) according to the directions of the manufacturer. Complementary oligonucleotides were synthesized with an *Eco*RI site on each end. The oligonucleotides were annealed and inserted into the 3.1-kb *Eco*RI

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TABLE 1. Bacterial strains

Bacterial strain	Description	Reference or source
B. megaterium		
VT1660		26
EG1312	VT1660(pEG204)	5
EG1724	VT1660(pEG424)	This study
EG1725	VT1660(pEG421)	This study
B. thuringiensis		
HD73-26	Crystal negative	6
HD263		7
EG1731	HD73-26(pEG421)	This study
EG1733	HD73-26(pEG424)	This study
EG1824	HD73-26(pEG438)	This study
EG1827	Spo$^-$ HD73-26	This study
EG1861	HD73-26(pEG23)	This study
EG1891	EG1827(pEG438)	This study
EG1899	EG1827(pEG23)	This study
EG7317	HD73-26(pEG452)	This study
EG7326	HD73-26(pEG204)	This study
EG7328	EG1827(pEG204)	This study
EG7329	EG1827(pEG452)	This study

fragment of pBC16 at the *Eco*RI site to create a multiple cloning site. The resulting plasmid pEG434 is shown in Fig. 1. The orientation of the inserted fragment was confirmed by restriction digestion and DNA sequencing.

Insertion of *cryIIA* and *cryIA(c)* into the *tet* expression vector. (i) pEG438. The 4.05-kb *Bam*HI-*Hin*dIII fragment from pEG201 was ligated to *Bam*HI- and *Hin*dIII-digested pEG434 (Fig. 1). The resulting plasmid pEG438 contains the *cryIIA* gene from pEG201 inserted into pEG434 downstream of and transcribed in the same direction as is *tet*.

(ii) pEG452. The 7.4-kb *Sph*I-*Nru*I fragment of *B. thuringiensis* DNA from pEG19 was ligated to *Sph*I- and *Nru*I-digested pEG434, resulting in pEG452 (Fig. 1). Plasmid pEG452 contains the *cryIA(c)* gene inserted into pEG434 downstream of and transcribed in the same direction as is *tet*.

Media. *B. megaterium* and *B. thuringiensis* strains were grown on NSM sporulation medium (13) when sporulation was required and on LB (16) or brain heart infusion plus 0.5% glycerol (BHIG) when a vegetative culture was required. *E. coli* strains were grown on LB. All cultures of plasmid-containing *Bacillus* strains were grown with 10 μg of tetracycline hydrochloride per ml. All cultures of plasmid-containing *E. coli* strains were grown with 25 or 50 μg of ampicillin per ml.

TABLE 2. Plasmids

Plasmid	Vector/toxin gene	Promoter from which toxin gene was transcribed	Reference or source
pEG19	pBR322/*cryIA(c)*		M. A. Von Tersch and H. Loidl Robbins, unpublished data
pEG201	pBR322/*cryIIA*		5
pBC16	pBC16/none		3
pEG23	pBC16/*cryIA(c)*	*cryIA(c)*	Von Tersch and Loidl Robbins, unpublished data
pEG204	pBC16/*cryIIA*	*cryIIA*	5
pEG421	pBC16/*cryIIA*	*cryIIA*	This study
pEG424	pBC16/*cryIIA*	*tet*	This study
pEG434	pEG434/none		This study
pEG438	pEG434/*cryIIA*	*tet*	This study
pEG452	pEG434/*cryIA(c)*	*tet*	This study

Construction of the *B. thuringiensis* sporulation-defective mutant EG1827. A 50-megadalton *B. thuringiensis* plasmid from HD119 (7) containing multiple *cryIA* genes was introduced by conjugation into crystal-negative *B. thuringiensis* HD73-26. This strain was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and a sporulation-deficient, crystal-negative mutant was identified. The presence of the 50-megadalton plasmid and the *cryIA* genes was verified by gel analysis and backcrossing to another crystal-negative strain. The strain was then cured of the 50-megadalton toxin plasmid by growth at high temperature, resulting in EG1827.

DNA manipulations. Restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., or Promega Biotec. Digestions and ligations were performed according to the directions of the manufacturers. Plasmids were isolated by the alkaline lysis procedure (16).

Transformation. Transformation of vegetative *B. thuringiensis* and *B. cereus* was by an adaptation of the electroporation procedure used for *Streptococcus faecalis* (Gary Dunny, personal communication). *B. thuringiensis* and *B. cereus* cultures were grown in BHIG. The cells were washed in EB (0.625 M sucrose, 1 mM $MgCl_2$) and suspended in one-third volume of EB. Cells (0.8 ml) were mixed with less than 10 μl of DNA in a 0.4-cm cuvette. A single discharge (2,500 V, 25 μF) from a Biorad Gene-Pulser was used for electroporation.

Transformation of *B. megaterium* protoplasts was by the method of Von Tersch and Carlton (26).

Transformation of *E. coli* RR1 and DH5α frozen competent cells from Bethesda Research Laboratories, Inc., was done according to the directions of the manufacturer. Transformation of *E. coli* GM2163-competent cells was by the $RbCl_2$ $CaCl_2$ method (16).

Sequencing. Sequencing of double-stranded plasmid DNA was done by the method of Sanger et al. (20), using Sequenase (U.S. Biochemical Corp.).

Western blot (immunoblot) immunoassay. Cells were harvested and suspended in 1/10 volume BSG buffer (0.85% NaCl, 0.03% KH_2PO_4, 0.06% Na_2HPO_4, 0.01% gelatin [Difco Laboratories]) plus 50 μM phenylmethylsulfonyl fluoride and frozen at −70°C. Samples were thawed, heated at 90°C to inactivate proteases, and treated with lysozyme (1 mg/ml) for 30 min to 2 h. If necessary, the samples were sonicated. Alternatively, pelleted cells of *B. thuringiensis* were suspended in mutanolysin (5,000 U/ml) and incubated for 30 min at 37°C. If necessary, sodium dodecyl sulfate (SDS) and NaOH were added to 6.2% and 0.12 M, respectively. For each sample, an equal number of cells (as determined by optical density at 600 nm) was added to Laemmli buffer and subjected to SDS-10% polyacrylamide gel electrophoresis (12). The gel was blotted onto nitrocellulose (23) and probed with polyclonal antibody raised in mice (24). Antibodies were raised to SDS-polyacrylamide gel electrophoresis-purified CryIA or CryIIA protein. The polyclonal CryIA antibody was raised against CryIA(b) protein and cross-reacts with CryIA(a), CryIA(b), and CryIA(c) protein (R. G. Groat, unpublished results). The secondary antibody was rabbit anti-mouse immunoglobulin G conjugated to alkaline phosphatase.

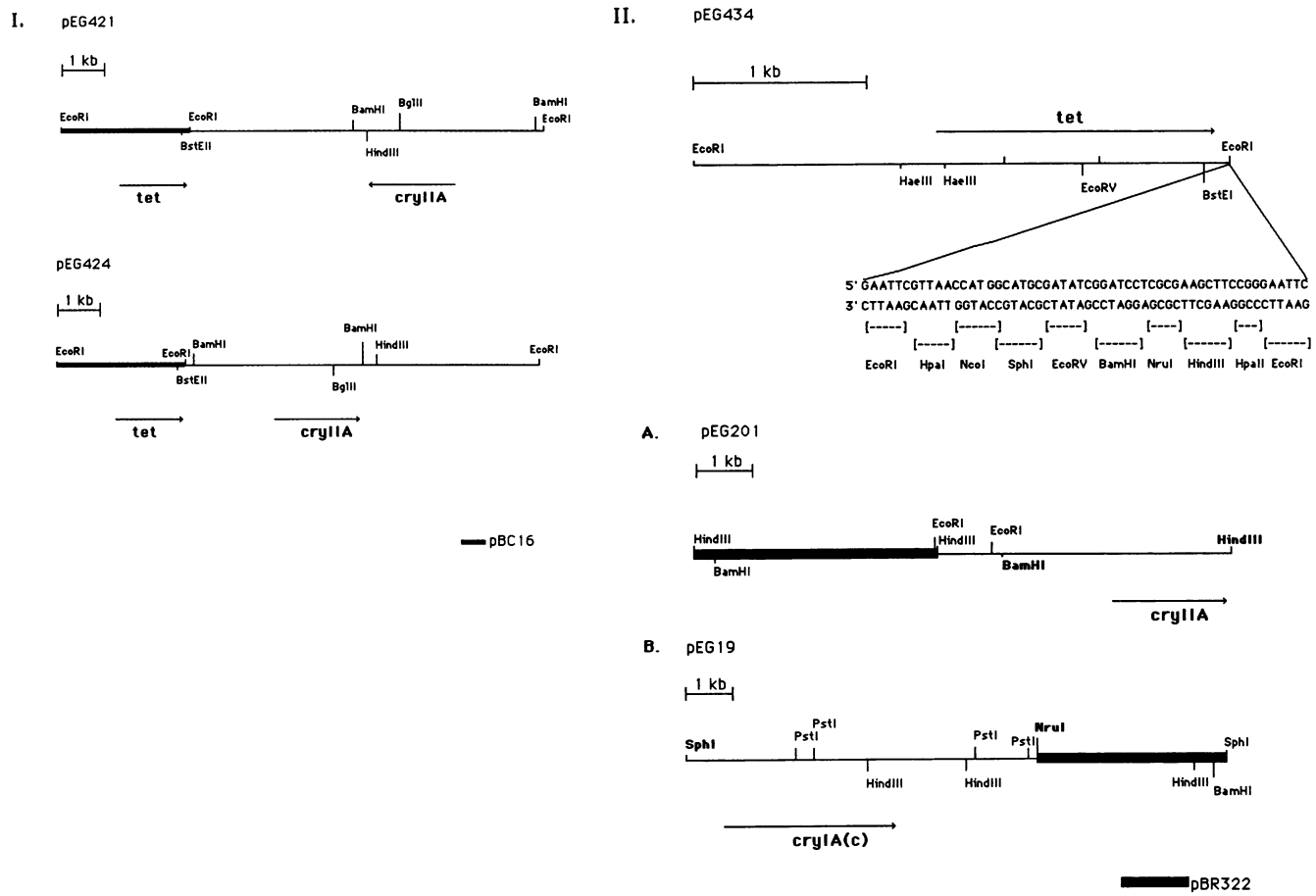

FIG. 1. (I) Linear restriction map of pEG421 and pEG424. Plasmids pEG421 and pEG424 contain the same *cryIIA* insert in opposite orientations in the 3.1-kb *Eco*RI fragment of pBC16. (II) Linear restriction map of pEG434 with the sequence of the multiple cloning site shown. The *Bam*HI-*Hin*dIII fragment (A) from pEG201 was inserted into the multiple cloning site of pEG434, resulting in pEG438. The *Sph*I-*Nru*I fragment (B) from pEG19 was inserted into the multiple cloning site of pEG434, resulting in pEG452.

RESULTS

Vegetative production of CryIIA δ-endotoxin from the *tet* promoter. A high level of β-galactosidase was produced during vegetative growth of *B. megaterium* from the *E. coli lacZ* gene (preceded by a *Bacillus* ribosome binding site) when it was cloned 3′ to the tetracycline resistance gene from the *Bacillus* vector pBC16 (data not shown). To determine whether insecticidal δ-endotoxin, which is normally produced only during sporulation, could be produced during vegetative growth by utilizing the *tet* promoter, the *B. thuringiensis cryIIA* gene was inserted downstream of the *tet* gene from pBC16. The *cryIIA* gene was inserted in both orientations downstream of the *tet* gene and transcribed in the same (pEG424) and opposite (pEG421) direction as was the *tet* gene. The *cryIIA* gene fragment that was inserted includes 1.9 kb of upstream DNA containing the *cryIIA* promoter and most of pBR322. These plasmids were introduced into *B. megaterium* VT1660 and *B. thuringiensis* HD73-26 via transformation.

B. megaterium VT1660(pEG424) produces large cuboidal crystals during stationary phase and small, round, phase-bright inclusions during vegetative growth. Western analysis indicated that CryIIA protein was produced during vegetative growth from pEG424 grown in NSM. In contrast, VT1660(pEG421), with *cryIIA* transcribed opposite to *tet*, showed no CryIIA until stationary phase (data not shown), as expected from regulation by the native *cryIIA* promoter.

B. megaterium VT1660(pEG424) and VT1660(pEG421) were also grown in LB plus glucose to determine whether it is possible to produce CryIIA protein under conditions that do not permit sporulation but support increased growth of the culture (Fig. 2). Samples were taken during mid- and late- logarithmic growth and in stationary phase. A spore count indicated that the cultures had not sporulated. The samples were Western blotted and probed with antibody to CryIIA. The strain containing pEG424 produced CryIIA protein throughout growth (Fig. 2, lanes 5 to 7). No CryIIA was seen from the *B. megaterium* strain which contained the *cryIIA* gene transcribed from the native promoter (pEG204) (lanes 2 to 4). A faint band of CryIIA was seen from VT1660 (pEG421) (*cryIIA* transcribed opposite to *tet*) (lanes 8 to 10), possibly because of a weak vector promoter. These results indicated that CryIIA can be produced, by utilizing the *tet* promoter, under conditions that inhibit sporulation.

Sequencing the 3′ end of the tetracycline resistance gene. Readthrough transcription from the *tet* promoter into genes cloned 3′ to the *tet* gene was not expected because Hoshino et al. (9) had sequenced a *Hae*III-*Eco*RI fragment from pTHT15 (which contains a *tet* gene similar to the *tet* gene from pBC16) and had found a possible transcriptional termi-

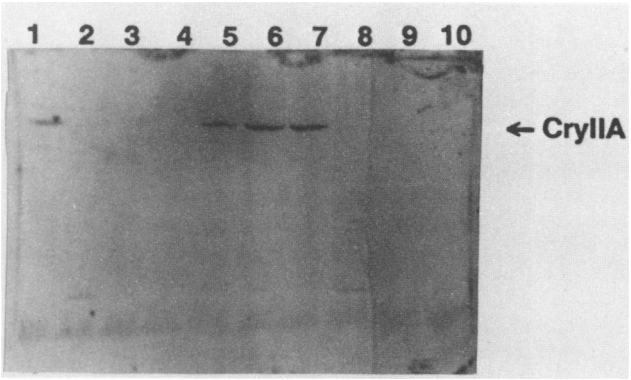

FIG. 2. CryIIA production from the *tet-cryIIA* fusion in the presence of glucose. *B. megaterium* strains containing pEG421, pEG424, and pEG204 were grown in NSM plus glucose (0.1%). Samples were run on an SDS–10% polyacrylamide gel and probed with antibody to CryIIA. Plasmids: pEG421, *cryIIA* transcribed opposite to *tet*; pEG424, *cryIIA* transcribed in the same direction as *tet*; pEG204, *cryIIA* transcribed from the native promoter. Lanes: 1, pEG204 (control, grown to sporulation in NSM); 2, pEG204 in mid-log growth; 3, pEG204 in late-log growth; 4, pEG204 in stationary phase (t = 15 h after the beginning of stationary phase); 5, pEG424 in mid-log growth; 6, pEG424 in late-log growth; 7, pEG424 in stationary phase (t = 15 h after the beginning of stationary phase); 8, pEG421 in mid-log growth; 9, pEG421 in late-log growth; 10, pEG421 in stationary phase (t = 15 h after the beginning of stationary phase).

nator 5′ to the *Eco*RI site used in the construction of pEG424 and the *tet-lacZ* fusion. If the *tet* gene from pBC16 consists of the same terminal sequences as that from pTHT15, transcription from the *tet* promoter would be expected to terminate prior to transcription of genes cloned into the *Eco*RI site.

To determine whether there are differences between these two *tet* genes that would allow transcription from *tet* into genes cloned into the *Eco*RI site of pBC16, the 3′ end of the *tet* gene from pBC16 was sequenced (Fig. 3). The sequence of the 3′ end of the gene 5′ to the *Eco*RI site was identical to that of the pTHT15 *tet* gene. The pBC16 *tet* gene contained the possible transcriptional terminator (with a free energy of

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5′ GGAATCATTG TCATTAGTTG GCTGGTTACC TTGAATGTAT ATAAACATTC
   GlyIleIleV alIleSerTr pLeuValThr LeuAsnValT yrLysHisSe

   TCAAAGGGAT TTCTAAATCG TTAAGGGATC AACTTTGGGA GAGAGTTCAA
   rGlnArgAsp PheEnd

   AATTGATCCT TTTTTTATAA CAGGAATTCA AATCTTTTTG TTCCATTAAA
                              EcoRI

   GGGCGCGATT GCTGAATAAA AGATACGAGA GACCTCTCTT GTATCTTTTT

   TATTTTGAGT GGTTTTGTCC GTTACATAGA AAACCGAAAG ACAATAAAAA

   TTTTATC 3′
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FIG. 3. Sequence of the 3′ end of the *tet* gene from pBC16. Underlined sequences indicate possible stem-loop structures. The *Eco*RI site used in cloning crystal protein genes is indicated. The free energy of formation for the stem-loop structure 5′ to the *Eco*RI site is $\Delta G = -12.4$. The free energy of formation for the stem-loop structure 3′ to the *Eco*RI site is $\Delta G = -16.2$.

formation for the stem of $\Delta G = -12.4$) that Hoshino et al. observed 5′ to the *Eco*RI site in the *tet* gene from pTHT15. However, in the sequence of the pBC16 *tet* gene 3′ to the *Eco*RI site, another possible terminator was found (with a free energy of formation for the stem of $\Delta G = -16.2$). The stem-loop structure that could form 3′ to the *Eco*RI site is probably a stronger terminator, and transcription might proceed through the first stem-loop structure and normally terminate at the stem-loop structure 3′ to the *Eco*RI site. When a gene is cloned into the *Eco*RI site, transcription from the *tet* gene continues into the inserted gene unless a transcriptional terminator is present in the upstream sequences of the inserted fragment.

Construction of an expression vector by using the *tet* promoter. To facilitate the cloning of different insecticidal δ-endotoxin genes, convenient restriction sites were inserted downstream of the *tet* gene (see Materials and Methods). The vector that was constructed, pEG434 (Fig. 1), contains the pBC16 origin of replication, the tetracycline resistance gene, and a multiple cloning site downstream from the *tet* gene. The multiple cloning site permits insertion of several different δ-endotoxin genes.

By using pEG434, it was possible to subclone the *cryIIA* gene without any pBR322 sequences by inserting the *Bam*HI-*Hin*dIII fragment from pEG201 into the *tet* expression vector (Fig. 1). The resulting plasmid (pEG438) was introduced into crystal-negative *B. thuringiensis* HD73-26. The resulting strain produces crystals during vegetative growth as well as during sporulation. HD73-26(pEG438) is toxic to *Lymantria dispar* and *Heliothis virescens* (data not shown). To confirm in *B. thuringiensis*, as was shown in *B. megaterium*, that CryIIA can be expressed from the *tet* promoter under conditions that do not allow sporulation, HD73-26(pEG438) was grown in BHIG for 16 h and assayed for the presence of CryIIA antigen by the Western immunoassay (Fig. 4A). Sporulation was inhibited under this growth condition. CryIIA was produced by HD73-26(pEG438) (Fig. 4A, lane 2) but not by the *B. thuringiensis* strain containing the *cryIIA* gene on a native plasmid (HD263) (lane 3) or HD73-26 carrying a cloned *cryIIA* gene expressed from its own promoter (pEG204) (lane 1). Figure 4A, lane 4, shows CryIIA protein produced from its own promoter on the native plasmid in a sporulated HD263 culture. The high-molecular-weight band that cross-reacts with the CryIIA antibody is consistently seen in this laboratory (5), and the nature of the protein is unknown.

Vegetative expression of CryIA δ-endotoxin from the *tet* promoter. To demonstrate the general utility of this expression vector for vegetative expression of insecticidal δ-endotoxins, the *cryIA(c)* gene from *B. thuringiensis* HD263 was subcloned onto pEG434 (Fig. 1). The *cryIA(c)* gene was subcloned downstream of the *tet* gene with the direction of transcription the same as that of the *tet* gene, resulting in pEG452. Plasmid pEG452 was introduced into *B. thuringiensis* HD73-26 via transformation. The resulting strain produces small, bipyramidal-shaped inclusions during mid-log phase and large bipyramidal inclusions during stationary phase. HD73-26(pEG452) is toxic to *H. virescens* and *Ostrinia nubialis* (data not shown). Western analysis of HD73-26(pEG452) indicated that CryIA protein was produced as early as mid-log phase (Fig. 5, lanes 5 to 8). In contrast, *B. thuringiensis* HD263 (lanes 1 to 4) containing *cryIA(c)* genes on native plasmids did not show any production of CryIA protein until stationary phase.

To determine whether *cryIA(c)* could also be expressed from the *tet* promoter under conditions that do not allow for

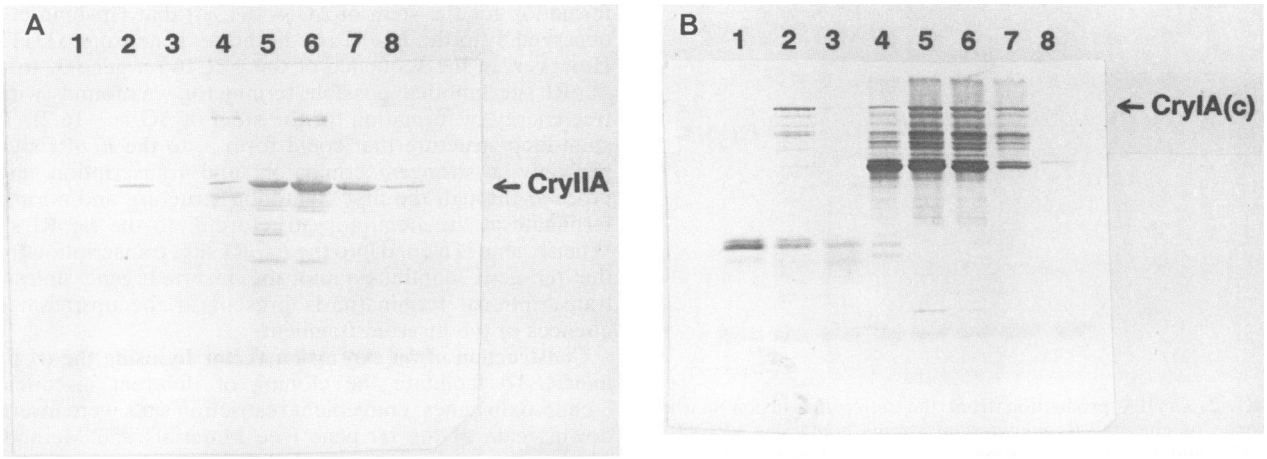

FIG. 4. Production of δ-endotoxin in *B. thuringiensis* in the absence of sporulation. (A) Western blot probed with antibody to CryIIA. In lanes 1 to 3, strains were grown in BHIG to inhibit sporulation; in lanes 4 to 8, strains were grown in NSM for 72 h to permit sporulation. Strains: lane 1, HD73-26(pEG204) contains *cryIIA* transcribed from its own promoter; lane 2, HD73-26(pEG438) contains *cryIIA* transcribed from the *tet* promoter; lane 3, HD263 contains *cryIIA* gene on native plasmid; lane 4, HD263 is sporulated; lane 5, HD73-26(pEG204); lane 6, HD73-26(pEG438); lane 7, EG1827(pEG438); lane 8, EG1827(pEG204). (B) Western blot probed with antibody to CryIA. In lanes 1 to 3, strains were grown in BHIG to inhibit sporulation; in lanes 4 to 8, strains were grown in NSM for 72 h to permit sporulation. Strains: lane 1, HD73-26(pEG23) contains *cryIA*(*c*) transcribed from the native promoter; lane 2, HD73-26(pEG452) contains *cryIA*(*c*) transcribed from the *tet* promoter; lane 3, HD263 contains *cryIA*(*c*) gene on native plasmid; lane 4, HD263 is sporulated; lane 5, HD73-26(pEG23); lane 6, HD73-26(pEG452); lane 7, EG1827(pEG452); lane 8, EG1827(pEG23). HD73-26 is Spo^+, and EG1827 is Spo^-.

sporulation, HD73-26(pEG452) was grown in BHIG for 16 h. The samples were Western blotted and probed with antibody to CryIA (Fig. 4B). The antibody reacted with a protein band from the strain containing the *tet-cryIA*(*c*) fusion (Fig. 4B, lane 2). Only a very faint CryIA band could be seen from HD263 (lane 3) or the strain containing cloned *cryIA*(*c*) transcribed from its own promoter [HD73-26(pEG23)] (lane 1). The small amount of CryIA production was probably due to a small number of cells which did sporulate. The lower-molecular-weight band that reacted with CryIA antibody was often seen in early vegetative samples of *B. thuringiensis* that do not contain any *cryIA* genes. CryIA protein was produced from the strain containing the *tet-cryIA*(*c*) fusion under conditions that would not allow sporulation and normally would not allow crystal production.

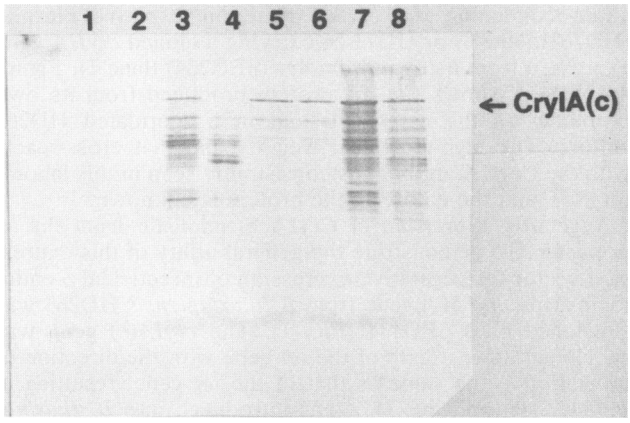

FIG. 5. Vegetative expression of CryIA δ-endotoxin from the *tet* promoter. *B. thuringiensis* HD73-26(pEG452) and a *B. thuringiensis* strain carrying the native plasmid containing the *cryIA*(*c*) gene (HD263) were grown in NSM. Samples were run on an SDS–10% polyacrylamide gel and probed with antibody to CryIA. Plasmid pEG452 contains *cryIA*(*c*) transcribed in the same direction as is *tet*. Lanes: 1, HD263 in mid-log growth; 2, HD263 in late-log growth; 3, HD263 is sporulating (t = 15 h after the beginning of stationary phase); 4, HD263 is completely sporulated (t = 29 h after the beginning of stationary phase); 5, pEG452 in mid-log growth; 6, pEG452 in late-log growth; 7, pEG452 is sporulating (t = 15 h after the beginning of stationary phase); 8, pEG452 is completely sporulated (t = 29 h after the beginning of stationary phase).

Expression from the *tet* promoter in a sporulation-defective mutant. To eliminate any effect of the native promoters, which are present on the *tet* fusion constructs, on expression during stationary phase and to further demonstrate that production of δ-endotoxin from the *tet* promoter does not depend on sporulation, the *tet-cryIA*(*c*) (pEG452) and the *tet-cryIIA* (pEG438) fusions were introduced into the *B. thuringiensis* sporulation mutant EG1827. This mutant is a derivative of HD73-26 that is blocked in sporulation before crystal production. Plasmids pEG23 [*cryIA*(*c*)] and pEG204 (*cryIIA*) were also introduced into EG1827. These strains were grown in NSM for 72 h, as were HD73-26 derivatives carrying the same plasmids. All HD73-26 derivatives were completely sporulated after 72 h. No spores were observed in the EG1827 derivatives. The samples were Western blotted and probed with antibody to CryIA (pEG23 and pEG452) or CryIIA (pEG204 and pEG438) (Fig. 4). Crystal protein was produced from pEG452 and pEG438, with *cryIA*(*c*) or *cryIIA* expressed from the *tet* promoter, in the Spo^- mutant. Only a very small amount of δ-endotoxin was produced from the native promoter (pEG23 or pEG204) in the Spo^- strains. The degradation of CryIA protein (Fig. 4B) is due to the action of proteases present during the long incubation period that was required to lyse the cells. The results indicate that expression of δ-endotoxin genes from the *tet* promoter has bypassed the dependence on sporulation for crystal production.

DISCUSSION

B. thuringiensis δ-endotoxins are potent insecticidal proteins. Their production depends on the ability of the cell to

sporulate and on growth conditions which permit sporulation. By using the expression vector pEG434, the production of δ-endotoxin no longer depends on sporulation. This expression system utilizes the promoter from the tetracycline resistance gene of pBC16 to direct transcription of cloned genes. By removing a potential transcriptional terminator, transcription from the *tet* gene continues into the inserted gene. This expression vector utilizes the replicon from the *Bacillus* vector pBC16 and can therefore be maintained in a *B. thuringiensis* host or in *B. megaterium* or other *Bacillus* species. Hoshino et al. (9) suggested, on the basis of sequence data, that the related *tet* gene from pTHT15 is inducible. If the *tet* gene from pBC16 is inducible, it would be possible to control the expression of genes cloned downstream of *tet*.

When *cryIA(c)* and *cryIIA* were subcloned into pEG434, both genes were expressed during vegetative growth in *B. thuringiensis*. Expression of *cryIIA* from the *tet* promoter was demonstrated in *B. megaterium*. In both species, δ-endotoxin production increased during sporulation. One explanation for the increased expression during sporulation is that the native promoters of both *cryIA(c)* and *cryIIA* are present on these constructs and initiated expression during sporulation, leading to an additive effect. No increase in β-galactosidase activity was seen upon sporulation when the *lacZ* gene was placed downstream of the *tet* gene (data not shown), supporting this hypothesis. However, the constitutive level of β-galactosidase expression from the *tet* gene was very high and would predict a higher level of vegetative expression of δ-endotoxin from the expression vector than that seen in *B. thuringiensis*.

The upstream DNA in the *tet-cryIIA* and *tet-cryIA(c)* fusions may contain regulatory sequences that decrease expression during vegetative growth. Schnepf et al. (21) found that expression of a δ-endotoxin gene in *E. coli* was modified by upstream DNA. If these sequences were deleted or displaced by the insertion of DNA sequences into this region, transcription of the δ-endotoxin gene increased. There might be upstream DNA on the *tet* fusion constructs that acted as a negative regulator on transcription during vegetative growth.

Another explanation is that there is regulation at the translational level. A 20-kilodalton protein has been found to increase expression of the *cytA* gene from *B. thuringiensis* subsp. *israelensis* (1). The 20-kilodalton protein was found to act in *E. coli* either during or after translation of the *cytA* gene. This translational regulation was seen in *E. coli* but has not been demonstrated in *B. thuringiensis*. Little is known about the regulation of native δ-endotoxin genes in *B. thuringiensis*, and additional transcriptional analyses are required to determine whether there is regulation at the translational level.

Native *B. thuringiensis* δ-endotoxin promoters showed no expression of δ-endotoxin under growth conditions that inhibited sporulation. When *cryIA(c)* and *cryIIA* were transcribed from the *tet* promoter, it was possible to express them under conditions that supported increased growth of the culture but suppressed sporulation.

When cloned δ-endotoxin genes were introduced into the *B. thuringiensis* sporulation mutant EG1827, very little crystal production was seen. However, when cloned toxin genes were expressed from the *tet* promoter, δ-endotoxin was produced in this Spo$^-$ mutant. The amount of δ-endotoxin produced during stationary phase was greater than that produced from the *tet* promoter during vegetative growth. Using the Spo$^-$ host eliminated the possibility of expression from the native promoter during stationary phase in the *tet* fusions and therefore rules out this explanation for the increase in expression during stationary phase. More crystal protein was made from the *tet* fusions in the Spo$^+$ host than in the Spo$^-$ host, indicating that the crystal gene promoter contributed to expression in an additive manner during sporulation.

With this expression system, production of δ-endotoxin is uncoupled from sporulation in two *Bacillus* species. The use of this expression vector is not necessarily limited to the expression of δ-endotoxin. This system could be useful for expressing other developmentally regulated genes during vegetative growth or for increasing the expression of genes that would normally be poorly expressed in *Bacillus* species.

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