Subspecies-Dependent Regulation of *Bacillus thuringiensis* Protoxin Genes

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Received 22 October 1998/Accepted 18 February 1999

*Bacillus thuringiensis* accumulates, primarily during sporulation, large quantities of insecticidal protoxins which are deposited as crystalline, intracellular inclusions. Most subspecies contain several plasmid-encoded *cry* genes, each of which has a unique specificity. The overall toxicity profile of a subspecies depends not only on the array of *cry* genes present but also on the relative expression of the genes. In general, transcription depends on sporulation-specific sigma factors, but little is known about regulation of expression of the individual genes. In order to determine whether expression of a particular *cry* gene varies in different subspecies, *lacZ* fusions to the *cry* promoters of two protoxin genes (*cry1* class) were constructed. Protoxin accumulation and mRNA contents were also measured by performing immunoblotting and Northern analyses, respectively. The expression of a *cry1Ab-lacZ* fusion, but not the expression of a *cry1C-lacZ* fusion, was three to four times lower in *B. thuringiensis* subsp. *aizawai* strains than in *B. thuringiensis* subsp. *kurstaki* or *B. thuringiensis* subsp. *aizawai* were lower. The regulation of the genes must involve regions upstream of the promoters which are unique to each *cry* gene since (i) mutations in the upstream region of the *cry1Ab* gene resulted in enhanced expression in *B. thuringiensis* subsp. *aizawai* and (ii) no differences were found when the *lacZ* fusions contained the *cry1Ab* promoters but no upstream sequences. The capacity to regulate each of the protoxin genes must be a factor in the overall protoxin composition of a subspecies and thus its toxicity profile.

**MATERIALS AND METHODS**

**Strains and growth.** The strains used and their origins are listed in Table 1. The presence of the *cry1Ab* gene in *B. thuringiensis* subsp. *kurstaki* HD1, *B. thuringiensis* subsp. *aizawai* HD133, *B. thuringiensis* subsp. *aizawai* HD112, and *B. thuringiensis* subsp. *tolworthi* HD124 had been established previously either by Southern hybridization (26) or by PCR analysis (11, 17, 18). Slot blotting with a specific *cry1Ab* oligonucleotide (17) was used to demonstrate that the relative amounts of *cry1Ab* DNA were the same in these subspecies (2, 3).

In order to establish that the regions upstream of the *cry1Ab* coding sequences were essentially the same in these subspecies, 1,025 bp of each sequence was amplified by PCR (27) by using oligonucleotides 5′GAAGACGG and 5′GGTTACTTAAACAATTATAAGG. The approximately 1-kb sequences upstream of the promoters of the three *cry1A* genes (*cry1Aa*, *cry1Ab*, and *cry1Ac*) in *B. thuringiensis* subsp. *kurstaki* HD1 were found to differ at only one base (14), and the sequence of the *cry1Ab* gene was confirmed (Fig. 1). Based on this sequence, restriction enzymes *Hpa*I, *Nsi*I, *Nde*I, and *Nol*I plus *Nde*I were used to demonstrate that the PCR digestion products of *B. thuringiensis* subsp. *kurstaki* HD1, *B. thuringiensis* subsp. *aizawai* HD133, and *B. thuringiensis* subsp. *tolworthi* HD124 were the same size.

The plasmid-cured strains 80-21, 5, and HD124-12 and the uncured strain HD112 served as hosts for the *lacZ* fusion plasmids. A cloned *cry1Ab* gene (8) was introduced by electroporation (28) into strains 80-21 and 5. This clone and all of the *lacZ* fusion plasmids were stable in the various strains, as judged by the constant level of resistance to chloramphenicol, the inability to find plasmid deletions after reisolation, and (for the *cry1Ab* clone) the extent of hybridization to the specific oligonucleotide probe in slot blots (2). Cells were grown in Luria broth (27) for preparation of DNA and in G-Tris medium (4) for all other experiments.

**Preparation of nucleic acids.** DNA and RNA were prepared (7, 8) from *B. thuringiensis* subsp. *kurstaki* HD1, strain 80-21, *B. thuringiensis* subsp. *aizawai* HD112 and HD133, and *B. thuringiensis* subsp. *tolworthi* HD124. RNA was isolated from cells 1 h after clumping (early sporulation with no visible endospores), about 1 h later when 40 to 50% of the cells contained phase-dull endospores, and after an additional 90 min when >80% of the cells contained phase-white to phase-bright endospores.

RNA was fractionated in an agarose gel for Northern blotting (27). A nitrocellulose filter was incubated with 35 pmol of 32P-labeled 5′GGATGCTCATAGAGGAGAAG, an oligonucleotide unique to the *cry1Ab* gene (17) which had been labeled by using [γ-32P]ATP and polynucleotide kinase (27). The X-ray films were scanned with a Phospholamger in order to determine relative amounts.

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lacZ fusions and β-galactosidase assays. A plasmid containing the promoter region of the cry1A gene with or without 280 bp upstream of the promoters fused to lacZ has been described previously (29). A 780-bp fragment upstream of the promoters of the cry1C gene (30) was introduced in the correct orientation as 280- or 780-bp HindIII fragments to the cry1A or cry1C wild-type promoters fused to lacZ (29, 32). The upstream region of the cry1A gene contains an inverted repeat (IR) and a potential bend sequence about 200 to 250 bp from the dual promoters (IR) and a potential bend sequence about 200 to 250 bp from the dual promoters (29) in order to establish the potential of the cry1C gene with or without 280 bp upstream of the promoters fused to lacZ has been described previously (29). A 780-bp fragment upstream of the promoters of the cry1C gene (30) was introduced in the correct orientation as 280- or 780-bp HindIII fragments to the cry1A or cry1C wild-type promoters fused to lacZ (29, 32). The upstream region of the cry1A gene contains an inverted repeat (IR) and a potential bend sequence about 200 to 250 bp from the dual promoters (Fig. 1) (30, 33).

Mutations in the −10 region of the BtII promoter (Fig. 1) which resulted in differences from the consensus sequence for both αα and ββ resulted in inactivation of the E promoter and at two bases for the BtII promoter. Neither of these −35 regions is highly conserved in Bacillus subtilis (35). However, the sequences differ substantially for at least 1 kb upstream of the promoters (Fig. 1) (30, 33).

In order to demonstrate that the lacZ fusion plasmids had not undergone any deletions or rearrangements, they were reisolated from B. thuringiensis strains. Consequently, β-galactosidase contents were confirmed by using subspecies which contained the same lacZ fusion plasmid. The lacZ fusion plasmids from B. thuringiensis were also transformed into Escherichia coli DH5α. These plasmids were digested with HindIII and BgIII (29) in duplicate, and the data obtained were converted to Miller units (22). The values agreed well, and equal quantities of spores (plus inclusions) were pelleted and then extracted (5). The inclusions were purified in Renografin gradients, and the protoxins were solubilized as described previously (5, 8).

Detection of protoxin antigens. Spores plus inclusions were harvested (usually after 24 h) and washed, and the relative spore concentrations were determined by direct counting in a Petroff-Hauser chamber (in triplicate) and by determining the absorbance at 600 nm. The values agreed well, and equal quantities of spores (plus inclusions) were pelleted and then extracted (5). The inclusions were purified in Renografin gradients, and the protoxins were solubilized as described previously (5, 8).

To prepare cell extracts, B. thuringiensis subsp. kurstaki HD1 and B. thuringiensis subsp. aizawai HD133 were grown at 30°C in 80 ml of G-Tris until about 50 or 80% of the cells contained phase-white to phase-bright endospores. At each point, 30 ml of cells was harvested, washed once with 10 ml of 1 M KCl-5 mM EDTA (pH 8.0) and twice with 10 ml of 0.1 M sodium dodecyl sulfate–5 mM dithiothreitol–2 mM phenylmethylsulfonyl fluoride (pH 9.6). The suspensions were then placed in a boiling water bath for 2 min. The protein contents were determined by using 5-μl portions and the bicinchoninic acid reagent (Pierce Chemical Co.). The samples were first precipitated in 1 ml of 10% trichloroacetic acid, and the pellets were dissolved in 0.2 ml of 0.2 M bicarbonate buffer, pH 10. The absorbance at 600 nm was determined with a Perkin-Elmer junior model 35 spectrophotometer until the cells became extensively clumped at the end of growth (at least when they were grown on glucose). β-Galactosidase assays were performed with 30- to 50-μl aliquots (29) in duplicate, and the data obtained were converted to Miller units (22). The specific activities were expressed in Miller units per unit of optical density at 600 nm, and the maximum values are reported below. These values are the averages of the values from at least three independent experiments, and the coefficients of variation were less than ±10% in all cases.

**TABLE 1.** B. thuringiensis strains used

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Protopine gene composition</th>
<th>Origin</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td>B. thuringiensis subsp. kurstaki HD1</td>
<td>cry1Aa, cry1Ab, cry1Ac, cry1IA, cry1HB</td>
<td>H. Dulmage, lab strain&lt;sup&gt;α&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>B. thuringiensis subsp. kurstaki 80-21</td>
<td>cry1Aa, cry1Ac, cry1IA, cry1HB</td>
<td>Plasmid-cured HD1</td>
<td>2, 7</td>
</tr>
<tr>
<td>B. thuringiensis subsp. aizawai HD133</td>
<td>cry1Aa, cry1Ac, cry1IC, cry1ID</td>
<td>NRL, lab strain&lt;sup&gt;β&lt;/sup&gt;</td>
<td>5, 17, 21</td>
</tr>
<tr>
<td>B. thuringiensis subsp. aizawai 5</td>
<td>cry1Aa, cry1Ac, cry1IC</td>
<td>Plasmid-cured HD133</td>
<td>2, 5, 7</td>
</tr>
<tr>
<td>B. thuringiensis subsp. aizawai HD112</td>
<td>cry1Ab, cry1IC, cry1ID&lt;sup&gt;γ&lt;/sup&gt;</td>
<td>NRL</td>
<td>2</td>
</tr>
<tr>
<td>B. thuringiensis subsp. tolworthi HD124</td>
<td>cry1Ab, cry1IC, cry1ID&lt;sup&gt;γ&lt;/sup&gt;</td>
<td>Plasmid-cured derivative</td>
<td>2</td>
</tr>
<tr>
<td>B. thuringiensis subsp. tolworthi 124-12</td>
<td>cry1IC, cry1E&lt;sup&gt;γ&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>α</sup> HD, original Howard Dulmage collection of strains.

<sup>β</sup> The strain has been maintained in the laboratory for a number of years.

<sup>γ</sup> NRL, Howard Dulmage collection kept by L. K. Nakamura, Northern Regional Laboratory, Peoria, Ill.

<sup>δ</sup> The gene composition may be incomplete (7).

FIG. 1. Sequence of the region upstream of the cry1A protoxin gene. The BtI and BtII promoters are underlined with one line and two lines, respectively. The start sites of transcription for BtI and BtII are indicated by I and II, respectively. The regions in boldface type are the potential bend and IR (arrows) sites of binding of the pyruvate dehydrogenase E2 protein (32, 33). The GenBank accession no. of this sequence is AF039908.
N NaOH. Equal quantities of protein were electrophoresed on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes for immunoblotting with a Cry1Ab monoclonal antibody plus an anti-rabbit alkaline phosphatase conjugate (27). After incubation with 35S-labeled oligonucleotides, immunodetection was performed with the iron-enhanced chemiluminescence method (30).

RESULTS

Subspecies variation in cry gene expression. Expression of the cry1A-lacZ fusion was three- to fourfold less in either of the two B. thuringiensis subsp. aizawai strains examined, strain 5 (derived from HD133), or HD112 than in B. thuringiensis subsp. kurstaki 80-21 or B. thuringiensis subsp. toluworthi HD124-12 when the organisms were grown in G-Tris supplemented with 0.1% glucose (Table 2). The lacZ fusion plasmid isolated from strain 5 had been used for transformation of strain HD124-12 and was also reintroduced into strain 80-21 in order to confirm the results. Similarly, the lacZ fusion plasmid from the original transformant of strain 80-21 was electroporated into strains 5, 80-21, HD124-12, and HD112, and the results were identical to those shown in Table 2. At the same time, these plasmids were transformed into E. coli DH5α in order to establish that there had been no major changes in the sizes of the plasmids or of the HindIII-BglII restriction fragments (29).

The differences observed with the cry1C-lacZ fusion were marginal (Table 2). This is a hybrid construct containing the cry1A-272 promoters plus the cry1C upstream region. Since the cry1A and cry1C genes have very similar dual overlapping promoter sequences (see above), such a construct should provide a valid assessment of the contribution of the upstream cry1C sequence to transcription in the various subspecies. The subspecies-specific responses of the cry1A and cry1C genes are likely to be due, therefore, to certain unique features of the upstream sequences. These sequences differ substantially in the cry1A (Fig. 1) and cry1C (30) genes.

Further evidence that these upstream sequences have a regulatory function included (i) the fact that the β-galactosidase specific activities for a fusion of only the cry1A promoters to lacZ in the absence of the upstream sequence were the same in strains 80-21 and 5 (Table 2) and (ii) the fact that mutations which substantially changed the potential bend region or the IR in the cry1A upstream sequence (Fig. 1) (33) resulted in somewhat lower specific activities in strain 80-21. However, the specific activities in strain 5 were the same as the specific activities in strain 80-21 and almost threefold higher than the specific activities in the wild type (Table 2). In the other B. thuringiensis subsp. aizawai strain, HD112, there was an approximately twofold increase in the specific activity due to the bend mutation. This strain had a different origin and perhaps a different cry gene composition than the other B. thuringiensis subsp. aizawai strain, HD133, and had not been cured of the plasmid containing the cry1Ab gene.

Measurements of cry1Ab mRNA. Total RNA from sporulating cells was fractionated in an agarose gel (27), transferred to nitrocellulose, and hybridized to a 32P-labeled oligonucleotide specific for the cry1Ab gene (Fig. 2). The relative contents of cry1Ab mRNA were 0.28:1.00 for HD133 and HD1 (Fig. 2, lanes 1 and 2) and 0.3:1.00 for HD112 and HD124 (lanes 3 and 4). The results obtained with RNA prepared from cells with >80% phase-dull to phase-white endospores are shown in Fig. 2, but the same differences were observed with RNA prepared at two earlier times during sporulation (see above). The differences in steady-state mRNAs were about the same as the differences found with lacZ fusions (Table 2), which confirmed that there were differences in transcription of this cry gene in the subspecies.

Measurements of Cry1Ab antigen. All of the subspecies which we studied contain several cry1 genes, including cry1Ab (Table 1). On the basis of the results obtained with the lacZ fusions, we anticipated that the amount of Cry1Ab antigen in B. thuringiensis subsp. aizawai inclusions should be less than the amount of Cry1Ab antigen in B. thuringiensis subsp. kurstaki or B. thuringiensis subsp. toluworthi inclusions. Proteins were extracted from purified inclusions and electrophoresed for staining or immunoblotting (Fig. 3). The total amounts of inclusion protein in the three subspecies were about the same (Fig. 3A), but there was considerably less Cry1Ab antigen in B. thuringiensis subsp. aizawai inclusions than in the inclusions of the other organisms (Fig. 3B). A Cry1A polyclonal antibody which also cross-reacted with other Cry1 proteins was used (Fig. 3C), and no major differences in the total protoxin antigen content were found.

The Cry1Ab antigen content of purified inclusions from B. thuringiensis subsp. aizawai HD133 (Fig. 3B) was considerably less than the content anticipated based on the measurements...
Subspecies-dependent differences in the expression of a cry1 gene were established with lacZ fusions and were confirmed by measuring cry1Ab mRNA and Cry1Ab protoxin antigen contents of sporulating cells and inclusion-spore extracts. In all cases, the cells were grown with glucose as the major carbon source. Similar differences were found when other carbon sources were used, although the absolute β-galactosidase specific activities differed (12).

The cry1Ab gene is present on a 40- to 50-kDa plasmid in all of the subspecies examined (2, 7). The copy numbers appear to be very similar based on hybridization in slot blots of total DNA with a cry1Ab-speciﬁc probe (2). In addition, differences in transcription were found with identical lacZ fusion plasmids, as well as with the same clone of the cry1Ab gene (Fig. 4B).

This subspecies-dependent regulation is attributable to the region upstream of the promoters since (i) there were no differences when only the promoters were fused to lacZ, (ii) fusion of the cry1C upstream region did not result in any difference, and (iii) the low level of transcription in strain 5 was enhanced by mutations in the potential bend and IR regions (Fig. 1) in the cry1A upstream sequence (Table 2). The segments used for mutagenesis were selected because a DNA binding protein identiﬁed as the E2 subunit of pyruvate dehydrogenase footprinted to these sites (32). There were decreases in the rates of β-galactosidase synthesis (as well as in the maximum speciﬁc activities, as shown in Table 2) in strains containing lacZ fusions with either the bend or the IR region mutated (32).

There were similar differences in steady-state cry1Ab mRNAs (Fig. 2), as well as in the relative accumulation of the Cry1Ab protoxin in sporulating cells, between B. thuringiensis subsp. kurstaki and B. thuringiensis subsp. aizawai (Fig. 4A). The amounts of this protoxin in sporulation extracts and especially in puriﬁed inclusions were somewhat variable. When Cry1Ab is the only protoxin produced, it is unstable, but it is stabilized by disulfide cross-linking to other protoxins in an inclusion (2, 23). Perhaps this protoxin does not cross-link as well with the Cry1C and Cry1D protoxins in B. thuringiensis subsp. aizawai as it does with the more closely related Cry1Aa and Cry1Ac protoxins in B. thuringiensis subsp. kurstaki and thus is more unstable in the former subspecies.

It is known that media can inﬂuence protoxin accumulation (13), so there may be catabolic properties unique to each subspecies which account for the differences in expression of the cry1Ab gene. This regulation may involve the relative amount of soluble E2 present in sporulating cells of each subspecies. This protein binds to cry gene upstream regions, and it could regulate transcription (32). Alternatively, or in addition, the protoxin compositions of the various subspecies may be a factor due to the competition among the cry genes for limiting transcription components (such as σ^E and σ^H). This possibility is difﬁcult to evaluate without information about the protoxin gene complement of each subspecies, the number of genes transcribed, and the extent of transcription of each gene (especially if the same sigma factors are used). Other unspeciﬁed subspecies differences may also inﬂuence the relative transcription of the genes. Whatever regulatory mechanism is involved, the gene-speciﬁc differences and the abilities of mutations in the cry1A upstream region to overcome low-level expression of the lacZ fusion in B. thuringiensis subsp. aizawai must be accounted for.

There are obvious practical implications for the differential expression of cry genes. Growth and sporulation conditions could affect the overall protoxin composition of inclusions and...
thus the toxicity profile. This regulation is likely to be significant to \textit{B. thuringiensis} in its natural environment, a possibility which is worth exploring.

**ACKNOWLEDGMENTS**

This research was supported by grant MCB-9600584 from the National Science Foundation and by Abbott Laboratories. Ping Cheng was a visiting scientist supported by the Ministry of Agriculture, People’s Republic of China.

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