APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1995, p. 4057–4060 Vol. 61, No. 11
0099-2240/95/$04.00+0

The Protoxin Composition of *Bacillus thuringiensis* Insecticidal Inclusions Affects Solubility and Toxicity

A. ARONSON*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received 5 June 1995/Accepted 12 September 1995

Most *Bacillus thuringiensis* strains producing toxins active on lepidoptera contain several plasmid-encoded δ-endotoxin genes and package related protoxins into a single inclusion. It was previously found that in *B. thuringiensis* subsp. *aizawai* HD133, which produces an inclusion comprising the CryIAb, CryIC, and CryID protoxins, there is a spontaneous loss in about 1% of the cells of a 45-mDa plasmid containing the *cryIAb* gene. As a result, inclusions produced by the cured strain were less readily solubilized at pH 9.2 or 9.5 and had a decreased toxicity for *Plodia interpunctella*, despite the presence of the CryIC protoxin, which was active when solubilized. These results suggested that protoxin composition was a factor in inclusion solubility and toxicity and that the *cryIAb* gene, which is also present on an unstable plasmid in several other subspecies, may have a unique role in inclusion solubility and toxicity. Introduction of a cloned copy of this gene into the plasmid-cured derivative of *B. thuringiensis* subsp. *aizawai* HD133 resulted in an increase in the solubility at pH 9.2 of all of the inclusion proteins from less than 20% to greater than 45% and a lowering of the 50% lethal concentration (LC_{50}, in micrograms [dry weight] per square centimeter) of inclusions for *Spodoptera frugiperda* from 35 to 10. These values are the same as those found with inclusions from *B. thuringiensis* subsp. *aizawai* HD133, and in all cases, the LC_{50} of the solubilized protoxins was 10. Transformants containing related *cryIA* genes produced inclusions which were more than 95% solubilized at pH 9.2 but also had LC_{50} of 10. The presence or absence of a particular CryIA protoxin is thus a major factor in inclusion solubility and toxicity. Since there is instability of a plasmid containing the *cryIAb* gene in many of these subspecies, the inclusions produced by a population of these cells must be heterogeneous in terms of solubility and thus toxicity. Such flexibility may be of adaptive value.

During the postexponential phase, *Bacillus thuringiensis* cells produce both a spore and one or two inclusion bodies. In the case of strains producing CryI protoxins primarily active on lepidoptera, the inclusions are made up of one or more disulfide cross-linked protoxins (6), each specific for a subset of these insects (15). The effectiveness of an inclusion obviously depends upon the activity spectra of the toxins (plus possible synergism) and its solubility in the larval midgut. For a particular inclusion, solubility varies with the insect (17) and also changes as a result of the spontaneous loss of a protoxin-encoding plasmid (2, 4).

For several well-characterized strains, the protoxin genes are confined to a few of the many plasmids often present in these isolates (12). In many cases, two or more of the genes are in close proximity on a plasmid with a size of >100 mDa (9, 24), and another gene, frequently *cryIAb*, is on an unstable plasmid with a size of 40 to 50 mDa (2). The instability is intriguing, because a significant difference between the CryIAb protoxin and related CryIA protoxins is the absence from the carboxyl half of the former of 26 residues, including four cysteines (11). This deletion affects not only the stability of this protoxin (10, 21) but probably its disulfide cross-linking with other CryI protoxins within an inclusion.

In *B. thuringiensis* subsp. *aizawai* HD133 (4) and in other subspecies (2), the spontaneous loss of a *cryIAb*-encoding plasmid resulted in inclusions which required a higher pH (in the presence of a sulfhydryl reagent) for complete solubilization. As a result, there was a lower toxicity of these inclusions for certain insects, such as *Plodia interpunctella*, despite the presence of an effective toxin (CryIC) in these inclusions (4). Solubilized protoxins were as active as the inclusions or soluble protoxins from the parental strain. The instability of a protoxin-encoding plasmid as well as the capacity to transfer these plasmids from cell to cell (12) is a mechanism for introducing heterogeneity into the properties of the inclusions produced by a population of these cells. If so, a key factor would be the unique properties of the CryIAb protoxin and the location of this gene on an unstable plasmid.

The special contributions of the CryIAb protoxin to inclusion properties were examined by transforming cloned copies of the *cryIAb* gene and of related *cryIA* genes into a strain which had spontaneously lost the *cryIAb*-encoding plasmid. The resulting inclusions were found to differ most extensively in their solubility properties, supporting a unique function for the CryIAb protoxin.

MATERIALS AND METHODS

Bacterial strains and growth. The relevant properties of various strains and of transformants are summarized in Table 1. Clones of the *cryIA* and *cryIC* genes are described below, and all were introduced by electroporation (25). The cells were grown in G-Tris medium (5) in a rotary shaker at 30°C for 6 to 7 h for the isolation of DNA, for various times between 15 to 20 h (20 to 80% with phase white endospores) for RNA (5), and for 24 to 40 h for complete sporulation and the isolation of inclusions. Strains containing clones of *cry* genes were grown in the presence of either 7 μg of chloramphenicol per ml or 25 μg of erythromycin per ml.

Plasmid constructs. A 7.1-kb *Bam*HI-*Pst*I fragment from *B. thuringiensis* subsp. *kurstaki* HD1 contained the *cryIAb* gene (11), and this fragment was cloned into an *Escherichia coli-B. thuringiensis* shuttle vector designated pTA (27). This cloning vehicle was constructed by first digesting pBD12 (13) with *Pvu*II in order to remove a nonessential 1.2-kb fragment from the 6.6-kb plasmid (14). The 322-bp *Pvu*II fragment containing the multiple cloning site from pUC18 was then introduced, and this was followed by a blunt-end ligation of the 1.87-kb *Ssp*I-*Pvu*II fragment from pUC18 into the *Sca*I site in the plasmid. The

* Mailing address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907. Phone: (317) 494-4992. Fax: (317) 494-0876.

TABLE 1. List of *B. thuringiensis* strains

Strain	Protoxin gene(s)	Relevant phenotype	Source or reference
HD133	*cryIAb*, *cryIC*, and *cryID*	Parental strain	USDA Northern Regional Lab
5	*cryIC* and *cryID*	Spontaneous loss of the 45-mDa plasmid	4
5/pTA-*cry*IAb	*cryIAb*, *cryIC*, and *cryID*	Clone of *B. thuringiensis* subsp. *kurstaki* HD1 *cryIAb* gene in strain 5	This study
5/pHT3101-*cry*IAc	*cryIAc*, *cryIC*, and *cryID*	Clone of *B. thuringiensis* subsp. *kurstaki* HD73 *cryIAc* gene in strain 5/pHT-*cry*Iab with loss of pHT-*cry*IAb	This study
5/pTA-pAC10	*cryIAb*, *cryIC*, and *cryID*	Clone of *B. thuringiensis* subsp. *alesti cryIAb* gene in strain 5	This study
CryB		Plasmid-cured derivative of *B. thuringiensis* subsp. *kurstaki* HD1	26
CryB/pTA-*cry*IAb	*cryIAb*	Clone of *B. thuringiensis* subsp. *kurstaki cryIAb* gene in CryB	This study
CryB/pHT3101-*cry*IAc	*cryIAc*	Clone of HD73 gene (as above) in CryB	This study
CryB/pHT3101-*cry*IC	*cryIC*	Clone of *cryIC* gene from strain 5 in CryB	This study

7.6-kb shuttle vector contained the ampicillin resistance gene from pUC18 and a chloramphenicol resistance marker for selection in *B. thuringiensis.*

The *cryIAc* gene from *B. thuringiensis* subsp. *kurstaki* HD73 (1) was cloned into shuttle vector pHT3101 (19) as an 8.4-kb *Sph*I-*Sal*I fragment. A 6.5-kb *Nde*I fragment containing the *cryIAb* gene from *B. thuringiensis* subsp. *alesti* (18) was also cloned into pTA. There are several largely conserved amino acid differences between the two CryIAb protoxins, but perhaps the most significant difference is the greater stability of the protoxin from subspecies *alesti*. This greater stability is due to the presence of 26 residues (including four cysteines) in the carboxyl half which are missing from the subspecies *kurstaki* CryIAb protoxin. There is a deletion of 78 bp between two direct repeats in the *cryIAb* gene from subspecies *kurstaki* HD1 (11) and subspecies *aizawai* HD133 (3) but not from subspecies *alesti.*

The *cryIC* gene was initially cloned from *B. thuringiensis* subsp. *aizawai* strain 5 as a 7-kbp *Eco*RI fragment (based on a partial restriction map [23]) in pUC18. An *Eco*RI digest of total strain 5 DNA was resolved in an agarose gel and a Southern transfer probed with the specific oligonucleotide shown below. A region of the gel, ca. 7 kb, was eluted and cloned into pUC18, and *E. coli* DH5α transformants were screened with the probe. The plasmid from a colony which produced a CryIC antigen with a size of ca. 130 kDa was used to subclone the 7-kbp *Eco*RI fragment into pHT 3101 for transformation into CryB (strain B/pHT3101-*cry*IC).

These clones were grown in *E. coli* DH5α, and alkaline minipreps (7) were used for electroporation (25). The pTA-*cry*IAb clone was transformed into strains 5 and CryB to produce strains 5/pTA-*cry*IAb and B/pTA-*cry*IAb (subspecies *kurstaki* gene). The pTA-pAC10 clone transformed into strain 5 resulted in strain 5/pTA-pAC10 (subspecies *alesti* gene). The pHT3101-*cry*IAc clone was electroporated into strains 5/pHT-*cry*IAb and CryB with selection for resistance to erythromycin. Colonies of strain 5/pHT-*cry*IAb transformants were then screened for loss of chloramphenicol resistance. The presence of each of these plasmids was confirmed by agarose gel electrophoresis (12) and DNA slot blots (see Fig. 2). Gene-specific oligonucleotides as described below were labeled with [γ-^{32}P]ATP and polynucleotide kinase (22) for hybridization with denatured DNA. The absence of the *cryIAb* gene in strain 5/pHT3101-*cry*IAc was confirmed by these procedures.

The oligonucleotides used for hybridization were 5′CAGAAGAATTGCTTT CATAGGCTCCGT for *cryIAb*, 5′TACCCCAATTAACGTTGAGGTGAATC GGGG for *cryIAc*, 5′TGTTAATACTATAACTCGTGC for *cryIC*, and 5′ATAG CACTTTCAGCAGCAG for *cryID*. Each labeled oligonucleotide was used for hybridization to either single-stranded DNA or to total RNA. Conditions for hybridization and washing were as previously described (5, 22).

Purification, toxicity, and solubility of inclusions. Confluent cultures were grown on G-Tris agar containing the appropriate antibiotic at 30°C for 36 to 40 h. Spores plus inclusions were scraped from the surface and washed, and inclusions were purified in gradients of Renografin (66% diatrizoate meglumine and 10% diatrizoate sodium; Solvay) as previously described (4, 5). The pure inclusions were dried in a Speed Vac (Savant), weighed, and stored at −20°C.

For bioassays of *Spodoptera frugiperda* larvae, the inclusions were suspended at a concentration of 1 mg/ml in 0.03 M $NaHCO_3$ and sonicated briefly to provide a homogeneous suspension. Various dilutions were prepared in 0.03 M $NaHCO_3$, and 0.1-ml portions were spread on the surface of diet cups (3 cm in diameter). The diet was the same as that used for *Heliothis virescens* bioassays (5). Eggs of *S. frugiperda* were obtained from Deryck Perkins, USDA Insect Biology Laboratory, Tifton, Ga. They hatched within 48 h when kept in a moist environment at 18°C. First or second instar larvae were used for bioassays, with 10 per dilution being employed. Mortality was determined after 7 days, and the data were analyzed (5). Each bioassay was repeated at least three times with separate preparations of inclusions, and the average values for three sets of experiments are reported.

Protoxin solubility was determined by suspending inclusions at a concentration of 1 mg/ml in deionized water and sonicating the mixture briefly. Portions were removed, and the inclusions were pelleted in a microcentrifuge. The conditions for extraction were based on those shown to be necessary for protoxin solubilization with retention of biological activity (16). Inclusions were initially extracted twice with 50 μl of 0.03 M Na_2CO_3–0.2% β-mercaptoethanol, pH 9.2, at 37°C for 20 min each time. The suspensions were centrifuged for 5 min in a microcentrifuge, and the supernatants were pooled. The pellets were then suspended in 0.03 M Na_2CO_3–0.5% β-mercaptoethanol, pH 9.6, and treated as described above. The final pellets were suspended in 0.2 N NaOH for protein measurements. Trichloroacetic acid was added to a portion of the extracts to a final concentration of 12%. After incubation on ice for 10 min, the precipitates were collected by centrifugation for 10 min in a clinical centrifuge, and the pellets were dissolved in 0.2 ml of 0.2 N NaOH. The protein contents were determined with the bicinchoninic acid reagent (Pierce Chemicals). Portions of these extracts were also fractionated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE), and the proteins were stained with Coomassie blue or transferred to a polyvinylidene difluoride membrane for immunoblotting with the CryIC antibody.

RESULTS

Construction of strains. About 1% of random colonies from cultures of *B. thuringiensis* subsp. *aizawai* HD133 grown at 42°C lacked the 45-mDa plasmid containing a *cryIAb* gene, as in strain 5 (4). In some cases, one or more of the other small cryptic plasmids was also missing. Strain 5 produced only *cryIC* and *cryID* mRNA (4) and thus presumably inclusions composed exclusively of the CryIC and CryID protoxins. The steady-state concentration of *cryIC* mRNA was about twice that of *cryID* mRNA (relative to gene copy numbers) in both strain 5 and the parental strain, HD133 (Fig. 1). These genes are in close proximity on the same large plasmid (23, 24), so neither plasmid locale nor copy number (Fig. 1) can account for this difference. The differences in relative amounts of steady-state mRNAs was confirmed by RNase protection assays with gene-specific oligonucleotides (3). It has previously been established that the half-lives of these mRNAs were 11 to

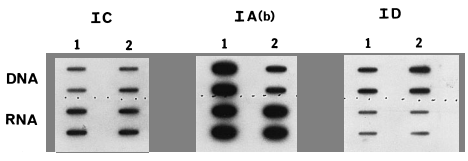

FIG. 1. Relative hybridizations of gene-specific oligonucleotides with RNA and DNA prepared from cells with ca. 60% phase white endospores of *B. thuringiensis* subsp. *aizawai* HD133 (lanes 2) and strain 5/pTA-*cry*IAb (lanes 1). Shown are slot blots (in duplicate) of 2 μg of denatured DNA and 10 μg of denatured RNA hybridized to gene-specific ^{32}P oligonucleotides as described in Materials and Methods.

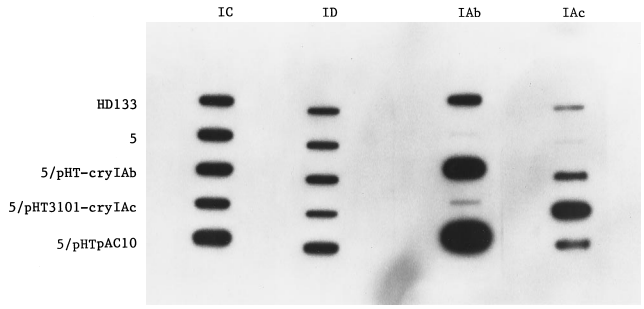

FIG. 2. DNA in slot blots hybridized to gene-specific oligonucleotides as indicated at the top of each lane. Each slot contained 2 μg of denatured DNA prepared from cells of the strains listed on the left. Under the conditions used, there was some cross hybridization of the *cry*IAc oligonucleotide with the *cryIAb* gene. The slots in the ID and IAc lanes are shifted slightly down relative to those in the other two lanes.

12 min (5), so these differences must be due to differences in the rates of transcription and presumably (if translational efficiencies are the same) in the amounts of the protoxins synthesized and packaged into inclusions (20). One of the consequences of the loss of a protoxin gene is the more extensive transcription of the remaining genes, as shown by the shifts in protoxin content (2) and by increases in the steady-state mRNA levels in strain 5 versus those in HD133 in RNase protection assays (3).

Various *cryIA* cloned genes were introduced into strain 5 or strain 5/pTA-*cry*IAb (Table 1) as described in Materials and Methods. The presence of the cloned genes was confirmed by slot blots with gene-specific probes (Fig. 2). These clones differed in copy number from the 45-mDa plasmid containing the *cryIAb* gene in the parental strain (Fig. 1), and they were more stable, with a frequency of loss of <1% (3). Despite the differences in gene copy numbers, the transcription of the three protoxin genes in strain 5/pTA-*cry*IAb was very similar to that in the parental strain (Fig. 1).

Inclusion solubility varies with protoxin composition. Active CryI protoxins can be solubilized from inclusions either in alkali at pH 11 to 12 or in a mercaptan buffer at a pH of >9.0. Bipyramidal inclusions from *B. thuringiensis* subsp. *kurstaki* HD1 which contain only CryIA protoxins were more than 80% solubilized in 0.2% β-mercaptoethanol at pH 9.2 (4), so these conditions were used initially for the solubilization of inclusion proteins from *B. thuringiensis* subsp. *aizawai* HD133 and its derivatives (Table 2). As noted previously, solubilization required a higher pH when the CryIAb protoxin was not present (strain 5), and the toxicity of such inclusions for *P. interpunctella* (4) and *S. frugiperda* (Table 2) was lower than that of inclusions from the parental strain. For strain 5 and all of the strain 5 transformants, the toxicity of inclusion proteins solubilized in 0.03 M Na_2CO_3–2.0% β-mercaptoethanol, pH 9.6, was the same as that of the inclusions or solubilized protoxins from strain HD133. The CryIC protoxin must be present in comparable amounts in these inclusions, since it is the only one among the two or three present in each inclusion which is effective on *S. frugiperda* (15).

Introduction of the cloned *cryIAb* gene (strain 5/pTA-*cry*IAb) restored the solubility of the inclusions and the toxicity for *S. frugiperda* to the parental strain, HD133 (Table 2). In contrast, inclusions from strains containing either the *cryIAc* gene or the *cryIAb* gene from subspecies *alesti* were virtually completely soluble at pH 9.2. These inclusions (and the solubilized protoxins) were as toxic for *S. frugiperda* as were those from the parental strain (Table 2).

TABLE 2. Solubility and toxicity of inclusion proteins

Source of inclusion[a]	Amt (%) solubilized[b]			50% lethal concn[c]
	pH 9.2	pH 9.6	0.2 N NaOH	
HD133	45	30	25	10 ± 2
5	15	40	45	35 ± 5
5/pTA-*cry*IAb	43	31	26	10 ± 2
5pHT3101-*cry*IAc	>90	0	<10	9 ± 3
5/pTA-pAC10	>80	<10	<10	11 ± 3
CryB/pTA-*cry*IAb	57	25	17	>100
CryB/pHT3101-*cry*IAc	80	9	10	>100
CryB/pHT3101-*cry*IC	15	52	32	9 ± 2

[a] Dried, purified inclusions were suspended at a concentration of 1 mg/ml in deionized water. Following a brief sonication, 0.2 ml was centrifuged in a microcentrifuge, and the pellets were sequentially extracted as described in Materials and Methods. One-third was precipitated with 12% trichloroacetic acid, and the pellets were dissolved in 0.2 ml of 0.2 N NaOH for bicinchoninic acid (Pierce) protein determinations. One-third was used for SDS-PAGE (Fig. 3 and 4).

[b] Extraction buffers and conditions are listed in Materials and Methods. These are average values for three separate experiments, with a range of ±2%.

[c] Units are micrograms of dry weight per square centimeter for *S. frugiperda* larvae, as described in Materials and Methods. The values are the averages of three separate experiments ± two standard deviations. In order to determine the protoxin content per microgram of dry weight, inclusions were solubilized in 6 M urea–50 mM dithiothreitol–1% SDS (pH 9.6) and fractionated by SDS–10% PAGE. The Coomassie blue-stained bands were quantitated with a Molecular Dynamics densitometer, with various concentrations of bovine serum albumin being used to construct a standard curve. The ca. 130-kDa protoxins were 65 to 70% of the inclusion dry weights in all cases.

The measurements of the fractions of the total inclusion proteins solubilized at each pH were confirmed by SDS-PAGE (Fig. 3). The solubilization of the CryIC toxin was monitored in immunoblots and was about the same as that of the total inclusion proteins (Fig. 4). There were clearly changes in the solubility of this protoxin which were dependent upon the presence or absence of other CryIA protoxins.

DISCUSSION

The presence or absence of a particular CryIA protoxin markedly affected the solubility of all of the inclusion proteins, presumably because of the extensive disulfide cross-linking among these related protoxins. The solubility properties differed even among inclusions containing closely related CryIA protoxins (Table 2). These differences would certainly affect the availability of a protoxin within the larval midgut and thus the overall effectiveness of a particular inclusion. Since the solubility and toxicity of inclusions from *B. thuringiensis* subsp.

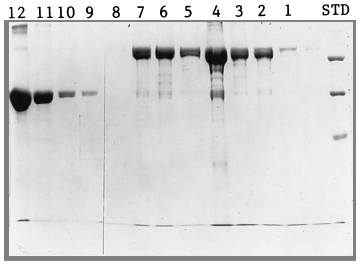

FIG. 3. Stained SDS–10% PAGE gel of aliquots of solubilized inclusion preparations (Table 2). Lanes 1 to 4, pH 9.2 extracts of strains 5, HD133, 5/pTA-*cry*IAb, and 5/pHT3101-cryIAc, respectively; lanes 5 to 8, subsequent pH 9.6 extracts of the inclusions (see Materials and Methods) from these same strains; lanes 9 to 12, 0.2, 0.5, 1.0, and 2.0 μg of bovine serum albumin used for the quantitation of the bands in lanes 1 to 8. Standards (STD) from top to bottom are β-galactosidase (113 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa).

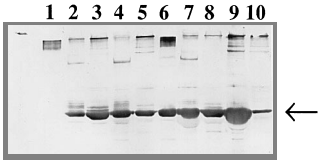

FIG. 4. Immunoblot with the CryIC antibody of the inclusion extracts listed in the legend to Fig. 3. Because of the instability of the CryIC protoxin, these extracts were dialyzed for 12 h at 4°C against 500 volumes of 0.03 M $NaHCO_3$ and then were digested with trypsin (1:25 for 2 h at 37°C). Lane 1, extract of CryB spores; lanes 2 and 3, pH 9.2 and 9.6 extracts of strain 5 inclusions (similar results were obtained with inclusions from strain B/pHT-*cry*IC); lanes 4 and 5, pH 9.2 and 9.6 extracts of strain HD133 inclusions; lane 6: 1 μg of pure CryIC toxin; lanes 7 and 8, pH 9.2 and 9.6 extracts of strain 5/pTA-*cry*IAb inclusions; lanes 9 and 10, pH 9.2 and 9.6 extracts of strain 5/pHT3101-*cry*IAc inclusions. The major band in each lane (arrow on the right) is ca. 60 kDa.

aizawai HD133, which has the *cryIAb* gene on a 45-mDa plasmid, and those from a strain containing a cloned copy of this gene (5/pTA-*cry*IAb) were the same, it is unlikely that factors encoded by the 45-mDa plasmid other than the CryIAb protoxin are involved in inclusion solubility properties.

The *cryIAb* protoxin gene is very prevalent among a variety of subspecies (8). It has been localized to a 40-to 50-mDa plasmid which is unstable in at least four subspecies (2). This class of *cryIAb* genes contains a 78-bp deletion between two direct repeats (11), and the 26 amino acids lacking from the CryIAb protoxin results in the instability of the protein (10, 21). As a result of this deletion, 4 of the 17 cysteine residues present in most CryI protoxins (located primarily in the carboxyl halves) are absent. There must be unique interactions of this protoxin with the others in an inclusion, which could account for the solubility properties intermediate between inclusions without the CryIAb protoxin and those containing other CryIA protoxins.

Inclusions from strains 5/pHT3101-*cry*IAc and 5/pTA-pAC10 are even more soluble at pH 9.2. These inclusions are as active on *S. frugiperda* as are those from the parental strain, but their greater ease of solubilization may result in a more rapid degradation and/or excretion of the toxins. The intermediate solubility properties of inclusions containing a CryIAb protoxin (lacking the 26 residues) could be a factor in extending the effective range of susceptible insects. Such a dual role for this protoxin may explain the widespread occurrence of the *cryIAb* gene (8).

It is surprising that inclusions produced by strain 5 are less active on two target insects despite the presence of the CryIC protoxin known to be effective when soluble. Inclusions from strain 5 are as toxic as the solubilized protoxins for *Manduca sexta* and *Trichoplusia ni* (3), implying that conditions in the midgut and thus the ease of solubilizing inclusions differ among various lepidopteran larvae (17). Inclusions composed of only the CryIC and CryID protoxins may be effective on other insects in which there are more favorable conditions for solubilization, or perhaps their effectiveness results from the slower solubilization of the protoxins.

Because of an unstable protoxin-encoding plasmid (which is maintained by cell mating [2, 12]), a population of *B. thuringiensis* subsp. *aizawai* HD133 cells produces at least two types of inclusions differing in protoxin composition, solubility, and toxicity. Such heterogeneity may provide flexibility for coping with a broad range of insects and could thus be an important adaptive mechanism for the survival of these bacteria.

ACKNOWLEDGMENTS

This research was supported by grant GM 34035 from the National Institutes of Health.

Lan Wu provided excellent technical assistance, and S. Mohammed provided the CryIC antibody.

REFERENCES

1. **Adang, M. J., N. S. Staver, T. A. Rocheleau, J. Leighton, R. F. Barken, and D. V. Thompson.** 1985. Characterized full-length and truncated plasmid clones of the crystal protein of *Bacillus thuringiensis* subsp. *kurstaki* HD73 and their toxicity to *Manduca sexta*. Gene **36:**289–300.
2. **Aronson, A. I.** 1994. Flexibility in the protoxin composition of *Bacillus thuringiensis*. FEMS Microbiol. Lett. **117:**21–28.
3. **Aronson, A. I.** Unpublished data.
4. **Aronson, A. I., E.-S. Han, W. McGaughey, and D. Johnson.** 1991. The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. Appl. Environ. Microbiol. **57:**981–986.
5. **Arvidson, P. E., H. Dunn, S. Strnad, and A. I. Aronson.** 1989. Specificity of *Bacillus thuringiensis* for lepidopteran larvae: factors involved *in vivo* and in the structure of a purified protoxin. Mol. Microbiol. **3:**1533–1543.
6. **Bietlot, H. P. L., J. Vishmulhatla, P. R. Carey, M. Pozsgay, and H. Kaplan.** 1990. Characterization of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis*. Biochem. J. **267:**309–315.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
8. **Bourque, S. N., J. R. Valéro, J. Mercier, M. C. Lavoie, and R. C. Leverque.** 1993. Multiplex polymerase chain reaction for detection and differentiation of the microbial insecticide *Bacillus thuringiensis*. Appl. Environ. Microbiol. **59:**523–527.
9. **Chambers, J. A., A. Jelen, M. Pearce-Gilbert, C. S. Jang, T. B. Johnson, and C. Gawron-Burke.** 1991. Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. J. Bacteriol. **173:**3966–3976.
10. **Geiser, M.** Personal communication.
11. **Geiser, M., S. Schweitzer, and C. Grimm.** 1986. The hypervariable region in the genes coding for entomopathogenic crystal proteins of *Bacillus thuringiensis*: nucleotide sequence of the kurd h1 gene of subspecies *kurstaki* HD1. Gene **48:**109–118.
12. **Gonzalez, J. M., Jr., B. S. Brown, and B. C. Carlton.** 1982. Transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Natl. Acad. Sci. USA **79:**6951–6955.
13. **Gryczan, T. J., and D. Dubnau.** 1978. Construction and properties of chimeric plasmids in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **75:**1428–1432.
14. **Hofemeister, J., M. Israeli-Reches, and D. Dubnau.** 1983. Integration of plasmid pE194 at multiple sites on the *Bacillus subtilis* chromosome. Mol. Gen. Genet. **189:**58–68.
15. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53:**242–255.
16. **Huber, H. E., P. Lüthy, H. R. Ebersold, and J.-L. Cordier.** 1981. The subunits of the parasporal crystal of *Bacillus thuringiensis*: size, linkage and toxicity. Arch. Microbiol. **129:**14–18.
17. **Jaquet, F., R. Hütter, and P. Lüthy.** 1987. Specificity of *Bacillus thuringiensis* delta-endotoxin. Appl. Environ. Microbiol. **53:**500–504.
18. **Lee, C.-S., and A. I. Aronson.** 1991. Cloning and analysis of δ-endotoxin genes from *Bacillus thuringiensis* subsp. *alesti*. J. Bacteriol. **173:**6635–6638.
19. **Lereclus, D., O. Arantes, J. Chaufaux, and M.-M. Lecadet.** 1989. Transformation and expression of a cloned delta-endotoxin gene in *Bacillus thuringiensis*. FEMS Microbiol. Lett. **60:**211–218.
20. **Masson, L., G. Préfontaine, L. Péloquin, and P. C. K. Lau.** 1989. Comparative analysis of the individual protoxin components in P1 crystals of *Bacillus thuringiensis* subsp. *kurstaki* isolates NRD-12 and HD-1. Biochem. J. **269:** 507–512.
21. **Minnich, S. A., and A. I. Aronson.** 1984. Regulation of protoxin synthesis in *Bacillus thuringiensis*. J. Bacteriol. **158:**447–454.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Sanchis, V., D. Lereclus, G. Menou, J. Chaufaux, S. Guo, and M.-M. Lecadet.** 1989. Nucleotide sequence and analysis of the N-terminal coding region of the Spodoptera-active δ-endotoxin gene of *Bacillus thuringiensis aizawai* 7.29. Mol. Microbiol. **3:**229–238.
24. **Sanchis, V., D. Lereclus, G. Menou, J. Chaufaux, and M.-M. Lecadet.** 1988. Multiplicity of delta-endotoxin genes with different insecticidal specificities in *Bacillus thuringiensis* aizawai 7.29. Mol. Microbiol. **2:**393–404.
25. **Schurter, W., M. Geiser, and D. Mathé.** 1989. Efficient transformation of *Bacillus thuringiensis* and *B. cereus* via electroporation: transformation of acrystalliferous strains with a cloned delta-endotoxin gene. Mol. Gen. Genet. **218:**177–181.
26. **Stahly, D. P., D. W. Dingman, L. A. Bulla, and A. I. Aronson.** 1978. Possible origin and function of the parasporal crystals in Bacillus thuringiensis. Biochem. Biophys. Res. Commun. **84:**581–588.
27. **Tsai, H., and A. I. Aronson.** Unpublished data.