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Cloning of the *nprA* Gene for Neutral Protease A of *Bacillus thuringiensis* and Effect of In Vivo Deletion of *nprA* on Insecticidal Crystal Protein

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The *nprA* gene, encoding *Bacillus thuringiensis* neutral protease A, was cloned by the use of gene-specific oligonucleotides. The size of neutral protease A deduced from the *nprA* sequence was 566 amino acids (60,982 Da). The cloned *nprA* gene was partially deleted in vitro, and the deleted allele, designated *nprA3*, was used to construct an *nprA3* strain (neutral protease A-deficient strain) of *B. thuringiensis*. Growth and sporulation of the *nprA3* strain were similar to those of an isogenic *nprA*$^+$ strain, although the extracellular proteolytic activity of the *nprA3* strain was significantly less than that of the *nprA*$^+$ strain. The *nprA3* strain produced insecticidal crystal proteins that were more stable than those of the isogenic *nprA*$^+$ strain after solubilization in vitro, and sporulated cultures of the *nprA3* strain contained higher concentrations of full-length insecticidal crystal proteins than did those of its isogenic counterpart. The absence of neutral protease A did not affect the insecticidal activity of a lepidopteran-specific crystal protein of *B. thuringiensis*. These results indicate that crystal protein stability and yield may be improved by deletion of specific proteases from *B. thuringiensis*.

During the process of spore formation, *Bacillus thuringiensis* synthesizes large amounts of certain proteins which aggregate to form crystals. These crystal proteins have been shown to be toxic to insects, especially those of the orders Lepidoptera (caterpillars), Coleoptera (beetles), and Diptera (mosquitoes) (for reviews, see references 2, 16, 19, and 22). Intact crystals (i.e., nonsolubilized) are resistant to proteolytic degradation. After ingestion by susceptible insects, the crystals are solubilized by the alkaline environment of the insect gut and the solubilized crystal proteins are proteolytically processed from full-length, inactive protoxins to smaller, active toxin fragments. Two sources of proteolytic activity have been identified that are capable of processing and/or degrading crystal protoxin: (i) proteolytic activity in the insect gut processes protoxin to active toxin (18, 21, 30, 42); and (ii) during sporulation, *B. thuringiensis* synthesizes proteolytic enzymes (8, 10, 28, 37), and this proteolytic activity may process and/or degrade crystal protein (1, 3, 7, 9, 23, 34). These studies showed that *B. thuringiensis* possesses several types of proteolytic activity; however, in most cases, the specific identities of the proteases of *B. thuringiensis* that process crystal protein have not been determined.

The goals of this study were to investigate the role of a specific protease of *B. thuringiensis* in cell growth and in crystal protein stability. A further goal was to determine whether a specific protease of *B. thuringiensis* could affect the insecticidal activity of crystal protein, since crystal protoxin must be proteolytically processed to yield active, insecticidal toxin (6, 46). We addressed these questions by cloning the *nprA* gene coding for neutral protease A of *B. thuringiensis* and disabling the gene in vivo. The effects of disabling *nprA* were determined with respect to total extracellular proteolytic activity, cell growth and sporulation, and crystal protein stability and toxicity.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *B. thuringiensis* HD strains were obtained from the collection of Dulmage (15). *B. thuringiensis* strains starting with the letters EG were isolated at Ecogen, Inc. EG10368 is a crystal-negative strain of *B. thuringiensis* that is readily transformable with plasmid DNA (14a). Plasmid pEG1111 contains the cloned *cry1Bb* crystal toxin gene (referred to as *cryET5* in reference 14) and directs the synthesis of the lepidopteran-toxic 130-kDa Cry1Bb protein during sporulation. Plasmid pEG272 contains the *cry3Bb2* crystal toxin gene [referred to as *cryIIIC*(*b*) in reference 13] and directs the synthesis of the coleopteran-toxic 73-kDa Cry3Bb2 protein during sporulation. *B. thuringiensis* cultures were grown in DSG sporulation medium [0.8% (wt/vol) Difco nutrient broth, 0.5% (wt/vol) glucose, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM $MnCl_2$, 10 μM $FeSO_4$] at 30°C for 3 to 4 days to permit sporulation and cell lysis to occur.

Protease activity and crystal protein quantification. *B. thuringiensis* cultures were grown in DSG medium at 30°C, and proteolytic activity in the culture supernatants was measured (33) at various times, with azoalbumin as a substrate, at pH 7.5. The relative amounts of crystal protein were determined by densitometer tracing of Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. Protein solubilization buffer consisted of 0.125 Tris-HCl (pH 7.5), 10% glycerol, 2% (wt/vol) SDS, 5% β-mercaptoethanol, and 0.2 mg of bromphenol blue per ml (final solution pH, 7.5).

DNA manipulations and cloning. *Escherichia coli* colonies containing plasmid libraries of *B. thuringiensis* DNA were constructed as previously described (12) by ligating size-selected *Pst*I or *Xba*I-*Bam*HI restriction fragments of *B. thuringiensis* EG2371 DNA into pUC18. The sequences of oligonucleotides WD205 and WD206 were based on residues 136 to 148 and residues 120 to 131, respectively, of the neutral protease of *Bacillus cereus* (35). The sequence of oligonucleotide WD205 is 5′-GGAATTGATGTAATTGGACATGAATTAACACATGCAGT-3′, and the sequence of oligonucleotide WD206 is 5′-ATGGTATATGGAGAA GGAGATGGAGTAACATTTAC-3′.

Insect bioassay. Sporulated cultures of *B. thuringiensis* containing known amounts of crystal protein were serially diluted in 0.005% (vol/vol) Triton X-100, and 50-μl aliquots of eight twofold serial dilutions were applied to the surfaces (175 mm^2) of artificial diets. Thirty-two neonate larvae (*Ostrinia nubilalis* and *Trichoplusia ni*) or third-instar larvae (*Plutella xylostella*) were tested on the diets at each dilution, and mortality was scored after 7 days.

Nucleotide sequence accession number. The sequence of the *nprA* gene has been deposited in the GenBank database under accession no. L77763.

RESULTS

Cloning and DNA sequence of *nprA*. Sidler et al. (35) reported the amino acid sequence of a 35-kDa (317-amino-acid) neutral protease from *B. cereus*. Based on the amino acid sequence of the *B. cereus* neutral protease, two oligonucleotide "guessmer" probes, designated WD205 (38 nucleotides) and

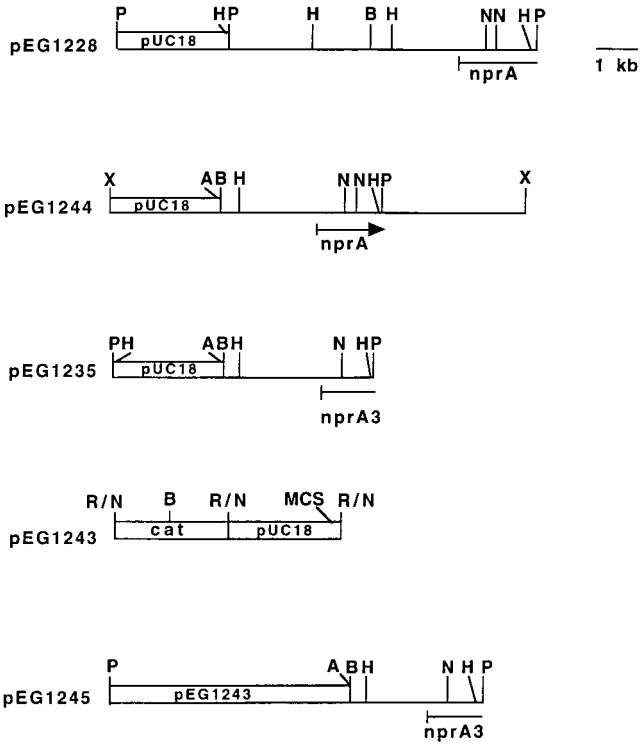

FIG. 1. Restriction maps of *nprA* plasmids. The letters above the maps represent restriction sites. Abbreviations: H, *Hin*dIII; N, *Nde*I; P, *Pst*I; B, *Bam*HI; A, *Asp*718; X, *Xba*I; R/N, blunt-end ligation of *Eco*RI and *Nde*I sites; MCS, multiple cloning site found in the pUC18 vector; cat, chloramphenicol resistance gene.

WD206 (35 nucleotides), were designed (see Materials and Methods). Radioactively labeled WD205 and WD206 each hybridized to an 8-kb *Pst*I fragment of DNA from *B. thuringiensis* subsp. *kurstaki* EG2371 in Southern blot experiments (data not shown). WD206 was used as a probe in colony hybridization experiments to isolate an 8-kb WD206-hybridizing *Pst*I fragment from a library of EG2371 DNA as part of a plasmid designated pEG1228 (Fig. 1). DNA sequencing revealed that the 8.0-kb fragment contained an *nprA* open reading frame which was truncated by a *Pst*I restriction site. The truncated *nprA* open reading frame was used as a probe in colony hybridization experiments in attempts to isolate the full-length *nprA* gene. In Southern blot experiments, the truncated *nprA* probe hybridized to a 7.5-kb *Bam*HI-*Xba*I DNA fragment (data not shown), and subsequently the fragment was cloned from a library of EG2371 DNA as part of a plasmid designated pEG1244 (Fig. 1). DNA sequencing confirmed that the 7.5-kb *Bam*HI-*Xba*I DNA fragment of plasmid pEG1244 contained the complete open reading frame of the *nprA* gene of *B. thuringiensis. E. coli* cells harboring pEG1244(*nprA*) grew slowly and yielded nonviable frozen cultures, suggesting that the cloned *B. thuringiensis nprA* gene was toxic to *E. coli.*

The nucleotide sequence of the *nprA* gene and the deduced sequence of the NprA protein are shown in Fig. 2. The sequence contains an open reading frame spanning nucleotides 182 to 1882 preceded by what is probably a ribosome binding site (Fig. 2). The first 18 amino acids of NprA resemble a signal peptide, with a short sequence containing 3 positively charged amino acids followed by 12 hydrophobic amino acids ending with an interruption of a potential beta strand, which conforms to the requirements for a typical signal peptidase recognition sequence (31). The *nprA* gene encodes a protein with a deduced size of 566 amino acids (60,982 Da). Thirty-four nucleotides downstream from the *nprA* stop codon is a perfect inverted repeat capable of forming a stable stem-loop structure ($\Delta G = -28$ kcal/mol as calculated by the rules of Tinoco et al. [40]) that may serve as a termination signal for transcription (47) and/or as an mRNA stabilization region (45). The deduced sequence of the NprA protein (Fig. 2) was found to be 99% identical to the deduced sequence of a 61-kDa neutral protease of *B. cereus* (44) and 38% identical to the 521-residue neutral protease of *Bacillus subtilis* (48). *B. cereus* and *B. thuringiensis* can be differentiated by several methods (4, 27), including determination of the lack of crystal protein production by *B. cereus*. The high degree of homology between the *B. thuringiensis* NprA protease and the *B. cereus* neutral protease (44) supports the proposal (3) that *B. thuringiensis* and *B. cereus* are closely related. The low degree of homology (38%) between the NprA protease and the *B. subtilis* neutral protease (48) is indicative of a distant relationship between these two bacterial species and suggests that the two proteases may perform different roles, although this has not been tested. The carboxyl half of the NprA protein, beginning at valine 250 (Fig. 2), was found to be 99% identical to the sequence of the purified 35-kDa neutral protease of *B. cereus* (35). Also beginning at valine 250, the carboxyl half of the NprA protein was found to be 74% identical to the purified thermolysin protease of *Bacillus thermoproteolyticus*, which was reported to contain 316 residues (41). These sequence homologies indicate that the active *B. cereus* neutral protease and the active *B. thermoproteolyticus* protease are derived by processing of larger precursors. Similar processing has been observed with the alkaline and neutral proteases of *Bacillus amyloliquefaciens* (43). Li and Yousten (24) purified and characterized a 37-kDa neutral protease from *B. thuringiensis* which, based on its size and level of activity, may be the processed form of NprA.

Deletion of *nprA*. The *nprA* gene contains three internal *Nde*I sites (Fig. 2). As a first step in the in vivo deletion of *nprA*, the gene was partially deleted in vitro by removing coding sequences between the *Nde*I sites. The 3.9-kb *Bam*HI-*Pst*I fragment containing the truncated *nprA* gene was purified from plasmid pEG1228 (Fig. 1), and the purified fragment was digested with *Nde*I. The digested fragment was ligated with pUC18 that had been double digested with *Bam*HI and *Pst*I. Transformation of the ligation mixture into *E. coli* and subsequent purification of plasmids from ampicillin-resistant colonies yielded a plasmid, designated pEG1235 (Fig. 1), that consisted of pUC18 plus a deleted form of the *nprA* gene, designated *nprA3*. The *nprA3* allele lacks 309 bp of sequence between the *Nde*I sites (Fig. 1).

A *B. thuringiensis* integration plasmid was constructed to permit integration of the *nprA3* allele into the *B. thuringiensis* chromosome. A 2.8-kb *Eco*RI fragment containing a chloramphenicol resistance determinant from plasmid pNN101 (29) was blunt-end ligated into the *Nde*I site of *E. coli* plasmid pUC18, yielding plasmid pEG1243 (Fig. 1). Properties of pEG1243 that permit it to be used as a *B. thuringiensis* integration vector are as follows: (i) pEG1243 replicates in *E. coli* but not in *B. thuringiensis*, (ii) pEG1243 contains a chloramphenicol resistance determinant functional in *B. thuringiensis*, and (iii) pEG1243 does not contain *B. thuringiensis* DNA sequences. Plasmid pEG1243 was digested with *Asp*718 plus *Pst*I, and the digested plasmid was ligated with the 3.6-kb *nprA3*-containing *Asp*718-*Pst*I fragment from pEG1235. The resulting plasmid, which consisted of pEG1243 plus *nprA3*, was designated pEG1245 (Fig. 1). Plasmid pEG1245 was transformed by electroporation (25) into the crystal-negative strain *B. thuringiensis* EG10368(*nprA*$^+$), and chloramphenicol-resistant colo-

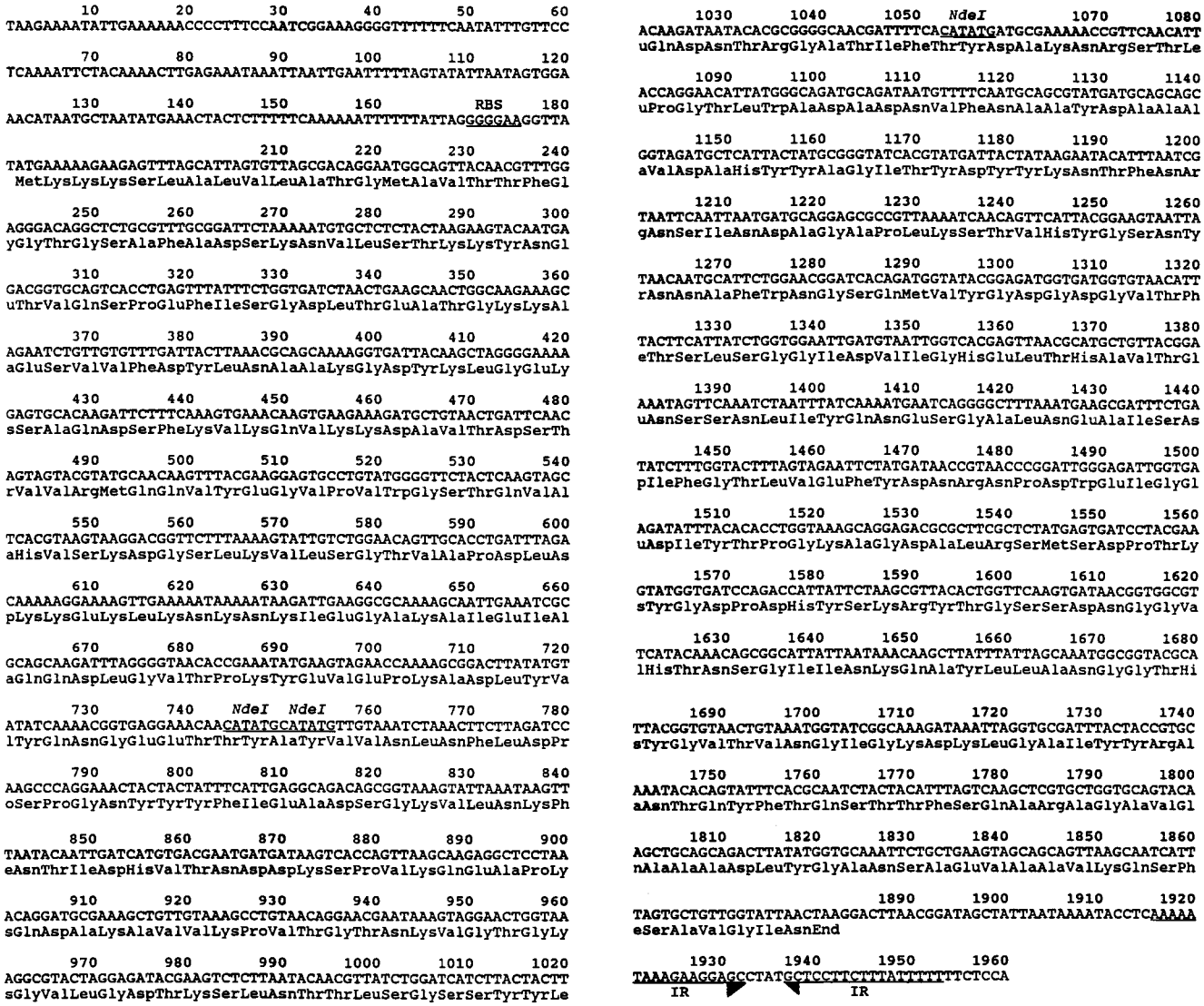

FIG. 2. DNA sequence of *nprA*. The complete sequence of the *nprA* gene is shown with the deduced sequence of the NprA protein. A putative ribosome binding site (RBS) at nucleotide 170 and *Nde*I restriction sites at nucleotides 746, 752, and 1055 are shown. An inverted repeat (IR) is indicated by underline arrows from nucleotides 1916 to 1953. Valine residue 250 (described in the text) is at nucleotide position 930.

nies were selected. It was expected that chloramphenicol-resistant colonies would arise only if pEG1245 integrated into the *B. thuringiensis* chromosome at the site of the *nprA* gene. After 2 days of incubation at 30°C, several transformant colonies formed on chloramphenicol-agar medium. Southern analysis revealed that, as expected, each of the chloramphenicol-resistant colonies contained both the *nprA* gene and the *nprA3* allele (data not shown). Three of the chloramphenicol-resistant colonies were grown in medium without chloramphenicol for approximately 30 generations to allow the loss of either the *nprA* gene or the *nprA3* allele by homologous recombination. After growth in medium lacking chloramphenicol, the cultures were diluted and plated for growth of individual colonies. Approximately 5% of the colonies were found to be chloramphenicol sensitive. Southern blot analysis of DNA from 16 chloramphenicol-sensitive colonies revealed that 12 of the 16 colonies contained *nprA*$^+$ but not *nprA3* and that 4 of the colonies contained *nprA3* but not *nprA*$^+$ (data not shown). One of the colonies containing *nprA3* was designated EG10624.

Biochemical and physiological effects of neutral protease A deficiency. Strains EG10368(*nprA*$^+$) and EG10624(*nprA3*) were grown in sporulation medium, and the cell densities and extracellular proteolytic activities of the cultures were measured. As shown in Fig. 3, the two cultures had similar, low levels of extracellular proteolytic activity during early logarithmic growth. As the cultures entered stationary phase, the proteolytic activity of the EG10368(*nprA*$^+$) culture increased from roughly twice that of the EG10624(*nprA3*) culture to over 10 times that of EG10624 (Fig. 3). During logarithmic growth, each strain had a doubling time of approximately 40 min (Fig. 3). The total cell counts and heat-resistant cell counts (spore counts) for the two strains were similar (Table 1).

Effects of neutral protease A deficiency on crystal protein. To measure the effect of neutral protease A deficiency on the insecticidal crystal protein, the crystal-negative strains EG10368 (*nprA*$^+$) and EG10624(*nprA3*) were transformed with plasmids carrying cloned crystal protein genes. In the first set of experiments, EG10368(*nprA*$^+$) and EG10624(*nprA3*) were trans-

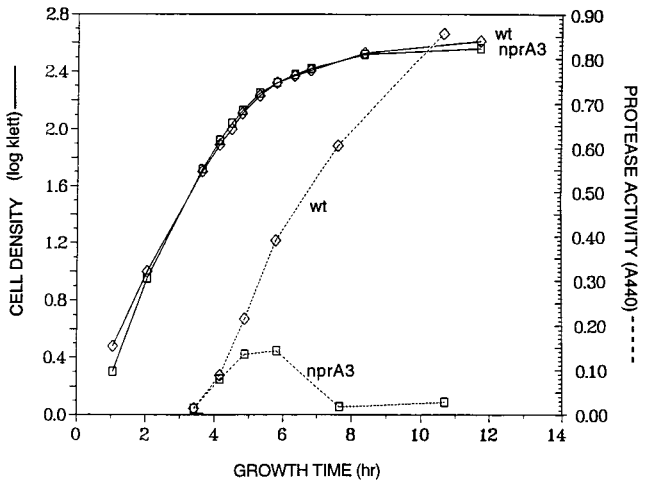

FIG. 3. Growth rates and proteolytic activities of *nprA*$^+$ and *nprA3* strains. Solid lines represent growth rates and dashed lines represent proteolytic activities of EG10368(*nprA*$^+$) (diamonds) and EG10624(*nprA3*) (squares).

formed with plasmid pEG272, which contains the cloned coleopteran-toxic *cry3Bb2* crystal protein gene (13), yielding strains EG10368(*nprA*$^+$ *cry3Bb2*$^+$) and EG10624(*nprA3 cry3Bb2*$^+$). The two strains were grown until sporulation and lysis had occurred, and Cry3Bb2 protein in the sporulated cultures was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The neutral protease A-positive strain EG10368(*nprA*$^+$ *cry3Bb2*$^+$) contained primarily the processed, 70-kDa form of the Cry3Bb2 protein (Fig. 4, lane 1). In contrast, the neutral protease A-deficient culture EG10624(*nprA3 cry3Bb2*$^+$) contained both full-length 73-kDa Cry3Bb2 and processed Cry3Bb2 protein (Fig. 4, lane 2).

The effect of neutral protease A deficiency on a lepidopteran-toxic crystal protein was studied by transformation of strains EG10368 and EG10624 with plasmid pEG1111, which harbors the cloned lepidopteran-toxic *cry1Bb* crystal protein gene (14), yielding strains EG10368(*nprA*$^+$ *cry1Bb*$^+$) and EG10624(*nprA3 cry1Bb*$^+$). EG10368(*nprA*$^+$ *cry1Bb*$^+$) and EG10624(*nprA3 cry1Bb*$^+$) were grown until sporulation and lysis had occurred, and Cry1Bb protein in the sporulated cultures was analyzed by SDS-PAGE. Repeated growth experiments showed that sporulated cultures of EG10624(*nprA3 cry1Bb*$^+$) contained from 1.2 to 1.5 times more full-length Cry1Bb crystal protein than sporulated cultures of EG10368 (*nprA*$^+$ *cry1Bb*$^+$) (Fig. 4, lanes 3 and 4).

It seemed likely that the increases in the amounts of full-length Cry1Bb and Cry3Bb2 crystal proteins that were observed in the neutral protease A-deficient strain were due to a decrease in the degradation of these crystal proteins in the *nprA3* background. As an indirect test of this possibility, we measured the rate of degradation of the Cry1Bb crystal protein from strains EG10624(*nprA3 cry1Bb*$^+$) and EG10368(*nprA*$^+$ *cry1Bb*$^+$) after the protein was solubilized in vitro. Sporulated cultures of the two strains were incubated at 45°C in protein solubilization buffer, conditions which would cause solubilization of the Cry1Bb crystal protein and which, it was believed, would permit proteases to remain active. Aliquots were removed from the incubation mix at various times and heated at 100°C to inactivate proteases. The amount of Cry1Bb in each aliquot was determined by SDS-PAGE. As shown in Fig. 5, Cry1Bb protein from EG10368(*nprA*$^+$ *cry1Bb*$^+$) was degraded in vitro after solubilization (lanes 2 to 6). A lower rate of degradation was observed for solubilized Cry1Bb from EG10624(*nprA3 cry1Bb*$^+$) (Fig. 5, lanes 8 to 12). The in vitro half-life of Cry1Bb was estimated by quantifying the amount of crystal protein remaining in each culture at various times after solubilization of Cry1Bb. The half-life of solubilized Cry1Bb was estimated to be 3 min in the neutral protease A-positive culture EG10368(*nprA*$^+$ *cry1Bb*$^+$) and 6 min in the neutral protease A-deficient culture EG10624(*nprA3 cry1Bb*$^+$). As expected, when proteolytic activity was minimized by washing each culture with 10 mM EDTA and then rapidly mixing each washed culture with 10 volumes of preheated (100°C) protein solubilization buffer, the neutral protease A-deficient culture EG10624(*nprA3 cry1Bb*$^+$) yielded approximately 1.3 times more Cry1Bb protein than the neutral protease A-positive culture EG10368(*nprA*$^+$ *cry1Bb*$^+$) (Fig. 5, lanes 1 and 7).

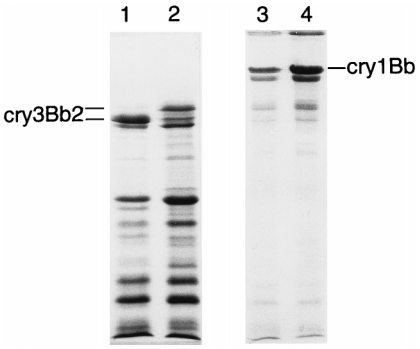

FIG. 4. Crystal protein production by neutral protease A-deficient and wild-type strains. Shown is a Coomassie blue-stained SDS-polyacrylamide gel in which were electrophoresed proteins solubilized from equal volumes of sporulated cultures of the following strains: EG10368(*nprA*$^+$ *cry3Bb2*$^+$) (lane 1), EG10624 (*nprA3 cry3Bb2*$^+$) (lane 2), EG10368(*nprA*$^+$ *cry1Bb*$^+$) (lane 3), and EG10624 (*nprA3 cry1Bb*$^+$) (lane 4). The lines indicate the positions of the Cry3Bb2 and Cry1Bb crystal proteins.

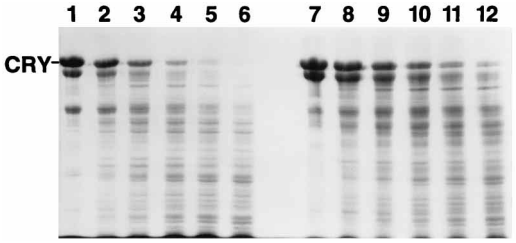

FIG. 5. In vitro degradation of Cry1Bb protein. A Coomassie blue-stained SDS-polyacrylamide gel is shown. Equal volumes of sporulated cultures of EG10368(*nprA*$^+$ *cry1Bb*$^+$) (lanes 1 to 6) and EG10624(*nprA3 cry1Bb*$^+$) (lanes 7 to 12) were incubated in crystal protein solubilization buffer at 45°C for 2 min (lanes 2 and 8), 5 min (lanes 3 and 9), 9 min (lanes 4 and 10), 14 min (lanes 5 and 11), or 20 min (lanes 6 and 12) prior to being heated at 100°C and loaded onto the gel. Lanes 1 and 7 contain equal volumes of EDTA-washed cultures of EG10368(*nprA*$^+$ *cry1Bb*$^+$) and EG10624(*nprA3 cry1Bb*$^+$) which were added immediately to 100°C-preheated protein solubilization buffer.

TABLE 1. Growth characteristics of *nprA*$^+$ and *nprA3* strains

Strain	Total cell count[a]	Spore count[b]	% Sporulation
EG10368(*nprA*$^+$)	$80 \times 10^7 \pm 7 \times 10^7$	$51 \times 10^7 \pm 5 \times 10^7$	64
EG10624(*nprA3*)	$83 \times 10^7 \pm 8 \times 10^7$	$58 \times 10^7 \pm 8 \times 10^7$	69

[a] The numbers are average cell counts per milliliter, ± standard deviations, calculated with data from at least three culture replications.

[b] Spore counts were determined after heating cultures at 65°C for 30 min.

TABLE 2. Effect of neutral protease A deficiency on insecticidal activity

Insect	PLC_{50} of Cry1Bb from[a]:	
	EG10368(*nprA*$^+$ *cry1Bb*$^+$)	EG10624(*nprA3 cry1Bb*$^+$)
O. nubilalis	112 (84–142)	98 (74–154)
P. xylostella	96 (63–136)	121 (89–158)
T. ni	6.0 (4.8–7.4)	5.9 (1.4–14.0)

[a] The numbers represent 50% protein lethal concentrations (i.e., the amounts [in nanograms] of Cry1Bb protein resulting in 50% insect mortality when applied to 175 mm^2 of insect diet surface). The numbers in parentheses are 95% confidence intervals.

Effect of neutral protease A deficiency on insecticidal activity. The effect of neutral protease A deficiency on the insecticidal activity of the Cry1Bb crystal protein was measured by applying dilutions of sporulated cultures of EG10368(*nprA*$^+$ *cry1Bb*$^+$) and EG10624(*nprA3 cry1Bb*$^+$), containing known concentrations of Cry1Bb, to the surfaces of diets for larvae of three species of lepidopterans: *Ostrinia nubilalis* (corn borer), *Plutella xylostella* (diamondback moth), and *Trichoplusia ni* (cabbage looper). Larval mortality was scored after 7 days. As shown in Table 2, for each of the lepidopteran species tested, the insecticidal activity of Cry1Bb from the neutral protease A-deficient strain EG10624(*nprA3 cry1Bb*$^+$) was similar to the insecticidal activity of Cry1Bb from the neutral protease A-positive strain EG10368(*nprA*$^+$ *cry1Bb*$^+$).

DISCUSSION

In this study, the role of a specific protease in the growth of *B. thuringiensis*, as well as in the stability and insecticidal activity of *B. thuringiensis* crystal proteins, was determined by displacing the gene for neutral protease A (*nprA*) with a disabled version of the gene, designated *nprA3*. This displacement was accomplished by the use of the integration plasmid pEG1245. In contrast to temperature-sensitive integration vectors that require elevated temperatures (>37°C) for homologous integration into a specific DNA site (49), pEG1245 integrates at any temperature since this plasmid is incapable of replicating in *B. thuringiensis*. Low-temperature integration is especially desirable for *B. thuringiensis* since it has been shown that growth of *B. thuringiensis* at elevated temperatures results in the loss of native, crystal protein-encoding plasmids (17). A disadvantage of pEG1245 is that larger amounts (one to several micrograms) of the plasmid are necessary to obtain *B. thuringiensis* transformants since the plasmid must enter the cell and, soon thereafter, integrate into the chromosome to yield a transformed, antibiotic-resistant cell.

The extracellular proteolytic activity of a *B. thuringiensis* strain containing the *nprA3* allele in place of the *nprA*$^+$ gene was found to be approximately 1/10 that of an isogenic *nprA*$^+$ strain when proteolytic activity was measured at pH 7.5 with azoalbumin as a substrate. The use of other substrates or pH values for this measurement could conceivably reduce the observed difference between the proteolytic activities of the *nprA3* and *nprA*$^+$ strains. With that possibility in mind, we note that no significant differences were found between an *nprA3* strain and an *nprA*$^+$ strain with respect to their rates of growth, final cell densities, and frequencies of sporulation. Similar results were reported for *Bacillus subtilis* by Yang et al. (48) and Kawamura and Doi (20), who found no difference between the sporulation frequencies of wild-type and protease-deficient strains of *B. subtilis*. It is logical that neutral protease A plays some role in cell growth and/or sporulation in *B. thuringiensis*, but that role could be masked by the growth conditions used in this study. Another possibility is that in the absence of neutral protease A, other *B. thuringiensis* proteases substitute for the function of neutral protease A. Examples of such enzyme substitution are seen with the RNA-processing enzymes RNAse II and polynucleotide phosphorylase. When either of these enzymes is inactivated in *E. coli*, growth is not affected; however, when both enzymes are inactivated, growth ceases (11). As discussed below, it is likely that *B. thuringiensis* produces several proteases and, if so, their activities may functionally overlap. This work demonstrates that neutral protease A contributes to the processing and degradation of *B. thuringiensis* crystal proteins. A *B. thuringiensis* strain containing the disabled *nprA3* allele in place of the wild-type *nprA*$^+$ gene produced lepidopteran-toxic Cry1Bb crystal protein that was degraded, after in vitro solubilization, at roughly one-half the rate of degradation of Cry1Bb protein produced by an isogenic *nprA*$^+$ strain. Furthermore, cultures of *nprA3* strains contained more full-length Cry1Bb protein and more full-length coleopteran-toxic Cry3Bb2 protein than cultures of isogenic *nprA*$^+$ strains. A related finding was reported by Thanabalu and Porter (38), who showed that strains of the mosquito-larvicidal bacterium *Bacillus sphaericus* that produced higher levels of proteolytic activity produced less mosquitocidal protein. However, the identities of the *B. sphaericus* proteases involved in the loss of the mosquitocidal protein were not known.

Our results suggest that a certain percentage of *B. thuringiensis* crystal proteins are susceptible to degradation by neutral protease A. In neutral protease A-deficient strains, this susceptible protein survives and is detected as increased full-length crystal protein. We attempted to minimize the in vitro proteolytic degradation of crystal protein, which occurs during SDS-PAGE analysis of crystal protein after the protein is solubilized from intact crystals, by washing cultures of *B. thuringiensis* spores and crystals with EDTA and by quickly inactivating proteases during crystal protein solubilization through rapid mixing of washed cultures with several volumes of hot (100°C) protein solubilization buffer. Despite these steps, we cannot rule out the possibility that some of the increased yield of full-length crystal protein seen in neutral protease A-deficient cultures was in fact due to decreased protein degradation in vitro during the processes of protein solubilization and SDS-PAGE analysis.

Crystal proteins display insecticidal activity when the crystal protoxin is proteolytically processed to an active toxin form. Proteolytic processing must be balanced with proteolytic degradation in order to yield maximum insecticidal activity; i.e., too much or too little proteolytic processing of crystal protein would result in a reduction in insecticidal activity. For example, MacIntosh et al. (26) found that trypsin inhibitors, when mixed with *B. thuringiensis* crystal proteins, increased the insecticidal activity of the proteins against target insects, indicating that a reduction in proteolytic activity could lead to increased toxicity. In contrast, Chilcott et al. (10) reported that increased levels of proteolytic activity corresponded with increased toxicity of certain *B. thuringiensis* strains against mosquito larvae. Alternatively, no correlation was seen between the level of proteolytic activity and the level of insecticidal activity of lepidopteran-toxic (1) or mosquito-toxic (32) strains of *B. thuringiensis*. Here we report that the toxicities of a lepidopteran-specific Cry1Bb protein produced by a neutral protease A-deficient strain of *B. thuringiensis* and of Cry1Bb produced by a neutral protease A-positive strain toward three species of caterpillars are similar. There are several possible explanations for the finding that neutral protease A deficiency did not affect insecticidal activity. (i) Results reported here showed that neu-

tral protease A was capable of degrading and/or processing the Cry1Bb protein. However, this degradation-processing may be unrelated to the type of proteolytic processing needed to form an active toxin. (ii) In the absence of neutral protease A, other *B. thuringiensis* proteases may function to activate the Cry1Bb protoxin to a toxin. As discussed below, *B. thuringiensis* probably produces several types of proteases that may be involved with crystal protein degradation and/or processing. (iii) A third possibility is indicated by the fact that proteolytic activity is present in the insect gut (18, 21, 30, 42), and this activity may be sufficient for the complete activation of protoxin to toxin in the absence of neutral protease A. At present we cannot confirm the validity of these possibilities.

Although the rate of degradation was reduced, the Cry1Bb protein from a neutral protease A-deficient strain was still degraded in vitro. Cry1Bb degradation in the absence of neutral protease A was not unexpected, since *B. thuringiensis* probably produces several proteases capable of degrading crystal protein. That *B. thuringiensis* produces multiple proteases has been suggested by studies using protease inhibitors, in which previous researchers found that proteolytic activities of the cysteine, metallo, and serine types were produced by *B. thuringiensis* (1, 3, 5, 9, 24, 32, 39). The possibility of multiple protease production by *B. thuringiensis* is also supported by analogy to *B. subtilis*, which has been shown to produce at least six distinct proteases (reference 36 and references therein). It may be that crystal protein stability can be maximized in *B. thuringiensis* when two or more protease genes are disabled. In addition to disabling the *nprA* gene, we are investigating the effect on crystal protein of disabling a *B. thuringiensis* alkaline protease gene.

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