APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1992, p. 642–646
0099-2240/92/020642-05$02.00/0

Vol. 58, No. 2

Insecticidal Properties of a Crystal Protein Gene Product Isolated from *Bacillus thuringiensis* subsp. *kenyae*

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Received 11 September 1991/Accepted 30 November 1991

A protoxin gene, localized to a high-molecular-weight plasmid from *Bacillus thuringiensis* subsp. *kenyae*, was cloned on a 19-kb *Bam*HI DNA fragment into *Escherichia coli*. Characterization of the gene revealed it to be a member of the CryIE toxin subclass which has been reported to be as toxic as the CryIC subclass to larvae from *Spodoptera exigua* in assays with crude *E. coli* extracts. To directly test the purified recombinant gene product, the gene was subcloned as a 4.8-kb fragment into an expression vector resulting in the overexpression of a 134-kDa protein in the form of phase-bright inclusions in *E. coli*. Treatment of solubilized inclusion bodies with either trypsin or gut juice from the silkworm *Bombyx mori* resulted in the appearance of a protease-resistant 65-kDa protein. In force-feeding bioassays, the purified activated protein was highly toxic to larvae of *B. mori* but not to larvae of *Choristoneura fumiferana*. In diet bioassays with larvae from *S. exigua*, the purified protoxin was nontoxic. However, prior activation of the protoxin by tryptic digestion resulted in the appearance of some toxic activity. These results demonstrate that this new subclass of protein toxin may not be useful for the control of *Spodoptera* species as previously reported. Hierarchical clustering of the nine known lepidopteran-specific CryI toxin subclasses through multiple sequence alignment suggests that the toxins fall into four possible subgroups or clusters.

The ability of *Bacillus thuringiensis* to produce insecticidal crystal proteins (ICPs) in the form of geometric crystalline inclusions during sporulation has been well documented (7, 11, 19). It has been shown that *B. thuringiensis* strains can harbor variable numbers of different ICP genes (22, 26). Since 1981, a variety of these ICP genes have been cloned and their gene products have been assayed against important insect pests, which revealed unique insecticidal spectra encoded by the various gene types (19, 32). As the relative proportion of protoxins can also vary within the intracellular crystal, it is now becoming possible to account for the extraordinary diversity in insecticidal spectra (18, 24, 37). A classification of ICPs based on their pathotypes and DNA sequence homologies has been recently proposed (19). Sequence comparisons of the different classes revealed a number of shared features, such as localization of cysteine residues primarily in the protease-sensitive carboxy-terminal region (33, 34), five highly conserved amino acid sequence blocks in the toxin region (19), and a potential membrane-spanning hydrophobic region in the N-terminal region (34). It has been postulated that the hypervariable regions found among the CryIA protoxins are responsible for the typical host range specificity of a given ICP (13). A report on hybrid genes by Ge et al. (12) has demonstrated such a specificity region for the *cryIA(a)* gene class which was localized between amino acid residues 332 and 450. Schnepf et al. (31) observed that more than one insect specificity-determining region can be found within the same gene.

Several groups have independently reported that ICPs may target a specific receptor(s) on the midgut cell surface; these data support the notion that ICPs possess a specific ligand domain (16, 21, 38, 40). Sequence comparison of new ICP genes having pathotypes similar to those of known ICPs but divergent sequences in the toxin region may help to identify consensus sequences characteristic of the different specificity domains. More information on the mode of action and functional domains of these ICP toxins will help to develop a basis for the design of more effective recombinant proteins.

In this paper, we describe the cloning and overexpression of a *B. thuringiensis* subsp. *kenyae* ICP gene and its toxicity to a variety of lepidopteran larvae.

MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* subsp. *kenyae* was obtained from the stock culture collection at the Forestry Pest Management Institute, Sault Ste. Marie, Ontario, Canada. *Escherichia coli* JM83 {*supE thi* Δ(*lac-proAB*) F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]} was used for cloning total genomic DNA isolated from *B. thuringiensis* subsp. *kenyae*. *E. coli* HB101 (*supE44 hsdS20* [r_B^- m_B^-] *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) was used for protein expression. Plasmids from *E. coli* were purified by the method of Birnboim and Doly (3). High-molecular-weight plasmids from *B. thuringiensis* were purified by the modified alkaline lysis protocol of Lereclus et al. (23).

Gene isolation. Total genomic DNA of *B. thuringiensis* subsp. *kenyae* was prepared by the method of Kronstad et al. (22). A genomic library was constructed by digesting *B. thuringiensis* subsp. *kenyae* DNA with *Bam*HI, ligation into *Bam*HI-digested pUC18, and transformation into *E. coli* JM83. Ampicillin-resistant colonies were screened by hybridization with a ^{32}P-labeled oligonucleotide probe (RB-18; 5'-AAT ACT TCC CAG AAA CC-3'). The isolated crystal protein gene was sequenced and published in a previous communication (4). Since the amino acid sequence (10) in

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the toxin region (i.e., amino acids 1 to 650) was most closely related to that of CryIC toxin (73.3% similarity) and then to those of the CryIA(c) and CryIA(b) toxins (71.0 and 70.5% similarity, respectively), we tentatively classified the gene in a new subclass called CryIC(b). However, an identical gene sequence, designated *cryIE*, was published simultaneously (39). We have agreed to reclassify our *cryIC(b)* gene as *cryIE* to eliminate any future confusion (38a).

Protein analysis and Western blotting (immunoblotting). ICP preparations from *E. coli* were made as described elsewhere (24). Total protein extracts from either *E. coli* harboring pMP40 or from inclusion bodies isolated from the same strain were examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and either stained with Coomassie brilliant blue or blotted onto nitrocellulose. To detect the recombinant protoxin expressed by the plasmid pMP40, polyclonal rabbit antibodies (provided by Anthony Pang, Forest Pest Management Institute), raised against *B. thuringiensis* subsp. *kurstaki* HD-1 crystals, were incubated with the blotted proteins for 16 h at 4°C and then with a protein A-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) for a further 2 h at 4°C. Localization of the primary antibody complex was visualized by the addition of 4-chloro-1-naphthol.

In situ gel hybridization. Total plasmid preparations isolated from *B. thuringiensis* subsp. *kenyae* were loaded on a 0.5% (wt/vol) agarose gel which was previously filtered and degassed. After electrophoresis, the gel was stained with ethidium bromide, photographed, and then dried under vacuum at 70°C. The dried gel was denatured and neutralized as described previously (26) and then hybridized with a ^{32}P-labeled specific oligonucleotide (5′-ATT TCT TCG AAG GAT ATC-3′) called 1488R. The 3′ end of 1488R is complementary to nucleotide 1488 in the *cryIE* gene sequence. The washed agarose gel was exposed for 24 h with Kodak X-Omat AR film by using an intensifying screen.

Insect toxicity assays. Insecticidal activities of recombinant inclusion body preparations against larvae of *Choristoneura fumiferana* Clemens (spruce budworm) and *Bombyx mori* L (silkworm), both from the rearing stock of the Forestry Pest Management Institute, were determined by using force-feeding experiments. The inclusion bodies were solubilized and activated before bioassay by using gut juice extracted from silkworm larvae by the procedure described by Gringorten et al. (15). The activated toxin solution was centrifuged, dialyzed in 50-kDa-molecular-size-cutoff tubing against 0.1 M CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer (pH 10.5), and passed through a 0.2-μm-pore-size low-protein-binding Acrodisc filter. The protein concentration of the activated toxin solution was determined by a microassay dye method (Bio-Rad) (5), and a twofold dilution series was prepared in 0.2 M CAPS buffer (pH 10.5). A 4-μl volume of each dilution was force-fed to 1-day-old sixth-instar spruce budworm larvae by using the technique described by van Frankenhuyzen and Gringorten (36). Effective dose estimates were obtained by probit analysis (27) of percent mortality observed after 48 h at 30°C in the silkworm assays (50% lethal dose [LD_{50}]) or of percent failure to reach pupation (PFD_{50}) after 10 days at 25°C in the budworm assays (36).

Insecticidal activities of recombinant CryIE inclusion body preparations against neonate *Spodoptera exigua* (Hübner) larvae (beet armyworm) were determined in diet incorporation assays as described by Moar et al. (25). Seven to eight concentrations were tested for each protein suspension. Each suspension was incorporated into artificial diet (containing no antibiotics and 0.1% formaldehyde). Mortality was observed after 7 days at 30°C. Approximately 20 insects were evaluated per concentration, and each treatment was replicated at least three times. To determine the insecticidal activity of the activated toxin against *S. exigua*, CryIE inclusion bodies were solubilized in 0.1 M CAPS buffer (pH 10.5) and incubated for 2 h with trypsin (final concentration, 0.5 mg/ml). The 65-kDa trypsin-resistant fragment was purified to homogeneity by anion-exchange chromatography and quantified by the method of Bradford (5). The activated toxin was incorporated into the media and tested as described above. However, only one discriminating concentration was used (50 μg/ml). This concentration was replicated three times.

Data were analyzed by using the Proc Probit procedure of SAS Institute, Inc. (30), after correction for control mortality by use of the formula of Abbott (1). Values from individual replicated experiments were pooled. Control mortality was <10%.

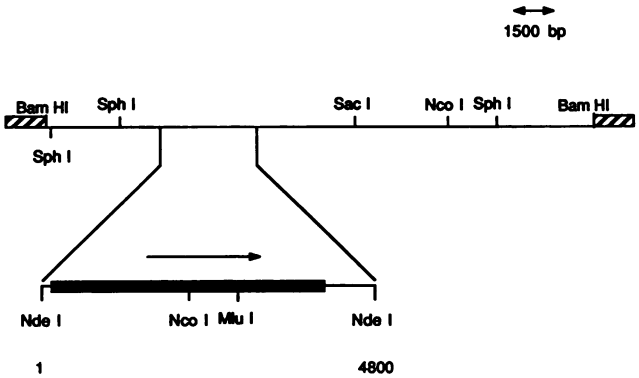

FIG. 1. Restriction map of pKEN4. The hatched end boxes represent pUC18 vector DNA. The dotted box represents the location of the gene on the large 19-kb *Bam*HI fragment, with the arrow indicating the orientation of the gene.

RESULTS

Cloning, expression, and immunodetection. Previous hybridization studies have shown that our 5′-terminal oligonucleotide probe RB-9, specific for *cryIA* genes, did not hybridize to *B. thuringiensis* subsp. *kenyae* total genomic DNA, although the oligonucleotide probe RB-18, specific for 3′ termini of known *cryIA* genes, could hybridize to an 8-kb *Hin*dIII fragment (26). Since this suggested the presence of a novel *cryIA*-like gene in this strain, the RB-18 probe was selected to screen the *B. thuringiensis* subsp. *kenyae Bam*HI genomic library. Approximately 1,200 colonies were screened, yielding two positive clones, both of which contained an identical 19-kb fragment. One clone, called pKEN4, was characterized further. Since the coding sequences of all known *cryIA* genes possess flanking *Nde*I sites, pKEN4 was subsequently digested with *Nde*I. The RB-18 probe hybridized to a 4.8-kb *Nde*I fragment in pKEN4 (Fig. 1), which was subsequently cloned into the expression plasmid pMPCV (24) for protoxin expression. A single plasmid clone, named pMP40, was selected for further study. *E. coli* cells harboring pMP40 expressed at high levels a 134-kDa protein which formed inclusion bodies (Fig. 2A, lanes 2 and 3). As shown in Fig. 2B (lanes 2 and 3), the protoxin responds to polyclonal antibodies raised against *B. thuringiensis* subsp. *kurstaki* HD-1 crystal. The same prep-

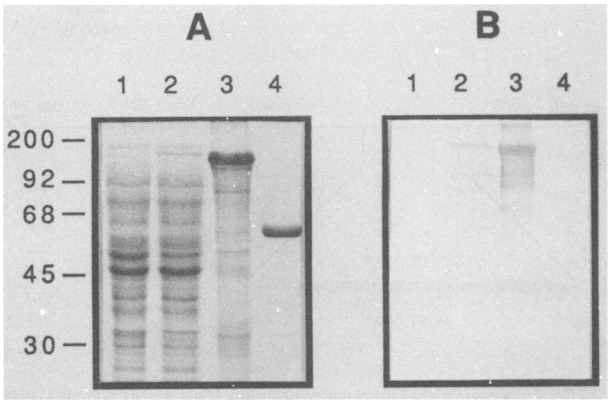

FIG. 2. Expression of the *B. thuringiensis* subsp. *kenyae* cloned protoxin gene. (A) SDS-polyacrylamide gel stained with Coomassie brilliant blue. Lanes: 1, total protein extract of *E. coli* HB101; 2, total protein extract of HB101 harboring the plasmid pMP40; 3, 5 μg of inclusion bodies purified from *E. coli* HB101 cells containing the plasmid pMP40; 4, purified 65-kDa trypsin-activated protoxin from inclusion bodies. Panel B is an immunoblot of an SDS-polyacrylamide gel identical to that shown in panel A but with polyclonal antibodies directed against crystals of HD-1.

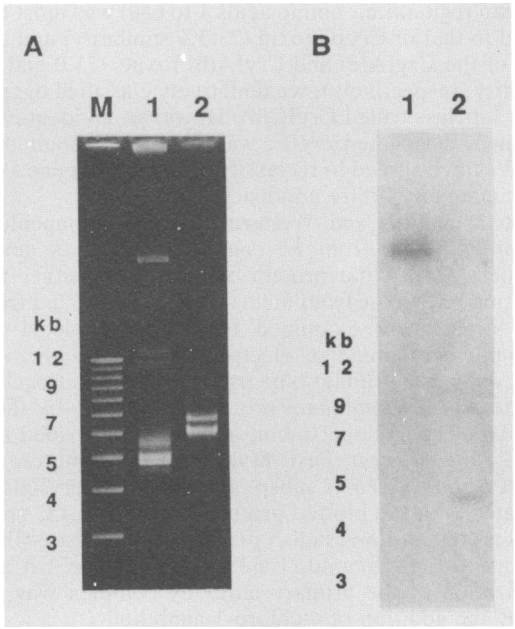

FIG. 3. Localization of *B. thuringiensis* subsp. *kenyae* protoxin gene. (A) Ethidium bromide-stained agarose gels containing a plasmid preparation from *B. thuringiensis* subsp. *kenyae* (lane 1) and a plasmid preparation of *B. thuringiensis* subsp. *kenyae* digested with *Nde*I (lane 2). (B) In situ DNA hybridization with the 1488R oligonucleotide probe of the dried agarose gel shown in panel A.

aration digested with trypsin (Fig. 2A, lane 4) or insect gut juice (not shown) generated a resistant peptide of 65 kDa, which corresponds to a typical toxin size for ICPs (11). Consistent with the hybridization data showing that the C-terminal end, but not the N-terminal region, is similar to that of the CryIA protoxins, the antibodies did not recognize the activated toxin (Fig. 2B, lane 4). This observation was further confirmed by determination of the pMP40 DNA sequence, the results of which were published elsewhere (4).

Gene localization in *B. thuringiensis* subsp. *kenyae*. It has been shown that crystal protein genes are generally located on high-molecular-weight plasmids (14, 23), although some chromosomal locations have been reported (28, 29, 35). To determine the location of the cloned 4.8-kb *Nde*I fragment, a total plasmid preparation from *B. thuringiensis* subsp. *kenyae*, in addition to an *Nde*I digest of the same preparation, was isolated and electrophoresed in an agarose gel (Fig. 3A). Only a single high-molecular-weight plasmid was observed which migrated slower than the faint, contaminating chromosomal band directly below it. This plasmid is estimated to be approximately 50 MDa in size. The dried agarose gel was probed in situ with the ^{32}P-labeled oligonucleotide 1488R as described in Materials and Methods. As shown in the autoradiogram in Fig. 3B, lane 1, the *B. thuringiensis* subsp. *kenyae* gene was localized to the single large plasmid seen in Fig. 3A. Furthermore, after plasmid digestion with *Nde*I, the oligonucleotide probe hybridized to a 4.8-kb band, verifying that it was this plasmid fragment which had been cloned into pMPCV.

Insect toxicity assays. The purified *B. thuringiensis* subsp. *kenyae* inclusion bodies from *E. coli* were examined for lepidopteran toxicity. The purified trypsin-activated CryIE toxin demonstrated a relatively high level of toxicity towards larvae of *B. mori*, having an LD_{50} of 60 ng per larva (Table 1). However, the recombinant gene product demonstrated poor toxicity against *C. fumiferana*, as demonstrated by a PFD_{50} of 1,824 ng per larva. On the basis of assays of more potent toxins, we know that the PFD_{50} measures a sublethal response at a toxin level that is about 20-fold lower than the LD_{50} in spruce budworm assays using activated CryIA toxins (36). The results observed with the purified *E. coli*-expressed CryIE toxin follow the same general trend when using crystal-spore mixtures from the actual *B. thuringiensis* subsp. *kenyae* strain which is believed to be a single lepidopteran gene strain (26). These mixtures were highly toxic towards the silkworm and poorly toxic towards the spruce budworm when compared with HD-1 and HD-73 controls (35a). Therefore, the results we observe seem to be inherent in the CryIE toxin and not dependent on whether the toxin is produced in *E. coli* or in *B. thuringiensis*. Since the *cryIE* gene product was reported to be highly toxic to spodopteran species (39), we examined the toxicity of the purified protoxin against larvae of *S. exigua*. Surprisingly, the purified CryIE protoxin was found to be nontoxic, whereas the larvae were highly sensitive to a purified recombinant *cryIC* protoxin gene product cloned from *B. thuringiensis* subsp. *aizawai* (HD-133) and expressed in *E. coli* (Table 2). To

TABLE 1. Toxicity of activated CryIE toxin against *C. fumiferana* and *B. mori*

Species	n^a	Slope (SE)	$ED_{50}{}^b$	95% Confidence limits		
				Lower	Upper	G value
C. fumiferana	120	2.3 (0.51)	1,824	1,069	2,631	0.188
B. mori	200	2.9 (0.39)	60	44	81	0.127

[a] Total number of larvae tested (20 to 30 per dilution)

[b] Effective dose (in nanograms of activated toxin per larva) estimates of the CryIE recombinant toxin against larvae of *C. fumiferana* and *B. mori*, expressed as the dose required to cause failure to reach pupation in 50% of the larvae (for *C. fumiferana*) or the dose required to cause 50% mortality (for *B. mori*).

TABLE 2. Comparative toxicity of *E. coli*-expressed protoxins against *S. exigua*

Gene type	n^a	Slope (SE)	$LC_{50}{}^b$	95% Confidence limits	
				Lower	Upper
cryIC	365	1.87 (0.47)	26	16	58
cryIE	372	ND[c]	>500		

[a] Total number of insects assayed (ca. 20 larvae per concentration)
[b] LC_{50} values in micrograms of protoxin per milliliter of diet.
[c] ND, not done.

ascertain whether the observed lack of CryIE toxicity was due to the inability of the insect to either solubilize or activate the recombinant protoxin, a trypsin-activated CryIE toxin was purified to homogeneity by ion-exchange chromatography and bioassayed at a discriminating concentration of 50 μg/ml. At this toxin concentration, the average *S. exigua* mortality observed among all three replicates was 34%.

DISCUSSION

An entomopathogenic gene, localized to a large 50-MDa plasmid in *B. thuringiensis* subsp. *kenyae*, was cloned and expressed at high levels in *E. coli*. This resulted in the production of a 134-kDa protein typical of CryI lepidopteran-specific protoxins. When examining the host range of the purified *cryIE* gene product by using three different insect species, larvae of *B. mori* but not of *C. fumiferana* or *S. exigua* displayed sensitivity to the toxin. In agreement with the in vivo toxicity results, we have also found that trypsin-activated CryIE toxin is nontoxic to cultured cell lines from either *C. fumiferana* or *S. exigua*, by using the lawn cell assay technique (15), thus distinguishing it from CryIC or CryIA(b) toxins (23a).

It is difficult to reconcile the differences between our CryIE toxicity results observed with *S. exigua* and those of Visser et al. (39). The explanation may rest with the method of bioassay used by Visser et al. (39), in which whole recombinant *E. coli* cells were utilized. By this method, the CryIE protoxin concentrations present in the diet cannot be quantitated. Furthermore, the toxic effects of cellular components from the bacterial cell itself, acting either directly or indirectly with the protoxin, are unknown. By overexpressing and purifying both the protoxin and the activated toxin, we have bypassed these potential problems to ensure that any toxic effects are due directly to the ingested protein. Another possibility for the differences in CryIE toxicity may be related to inherent susceptibility differences between the different insect colonies. However, our colony of *S. exigua* appears to be similar to that of Visser et al. (39) in that both are highly sensitive to the CryIC toxin.

It has been shown by Jaquet et al. (20) that, in certain cases, solubilization and/or preactivation of the protoxin could greatly increase the potency of a poorly toxic protoxin. Therefore, the possibility that the crude *E. coli* extracts utilized by Visser et al. (39) had proteolytically activated the protoxin to toxin via endogenous proteases could be eliminated by directly testing purified activated toxin. Indeed, it was observed that the C-terminal moiety of the protoxin played a role in masking the toxicity of the protease-resistant toxin. Although prior removal of the protease-sensitive C terminus of the CryIE protoxin resulted in the appearance of toxic activity towards *S. exigua*, the level of activity was poor in comparison to that of the CryIC protoxin. In summary, we have found that the *cryIE* gene class is relatively ineffective against *S. exigua*.

Since a large number of new genes from *B. thuringiensis* have been cloned and sequenced within the past few years (4, 8, 13a, 17, 39), it was of interest to compare the primary amino acid sequences of all known lepidopteran-specific CryI toxins to date to delineate how the proteins are interrelated. For that purpose, the MULTALIN software (9) was used to calculate hierarchical clustering and consensus sequence. The dendrogram in Fig. 4 shows the relatedness among the activated toxins (i.e., the first 650 amino acids). Interestingly, two main toxin clusters can be found. The first cluster contains the three CryIA toxins, with the CryIA(a) and CryIA(b) being the most closely related. The CryIF, CryIC, CryIE, and CryID toxins are found in a separate cluster, with the CryIF being the most closely related and CryID being the least closely related to the CryIA cluster. One observation which supports the close relationship between these four subclasses is that all four have demonstrated toxicity towards *Spodoptera* species (8, 17, 29). Another interesting feature found within this cluster is that CryIC is linked to the CryIE toxin. This feature is consistent with our results which have shown that the activated toxin but not the protoxin possesses some toxicity towards *S. exigua*. The CryIB toxin is distantly related to the two toxin clusters, with CryIG showing the least relatedness to all of the known toxin classes. Since CryIB and CryIG appear not to be closely related either to the other clusters or to each other, they may each form the nucleus of a new cluster. As more toxin sequences become available, it will be interesting to observe whether new clusters can be created on the basis of sequence and pathotype similarity for each of the CryIB and CryIG subclasses.

ACKNOWLEDGMENT

We thank Al Mazza for his excellent technical assistance.

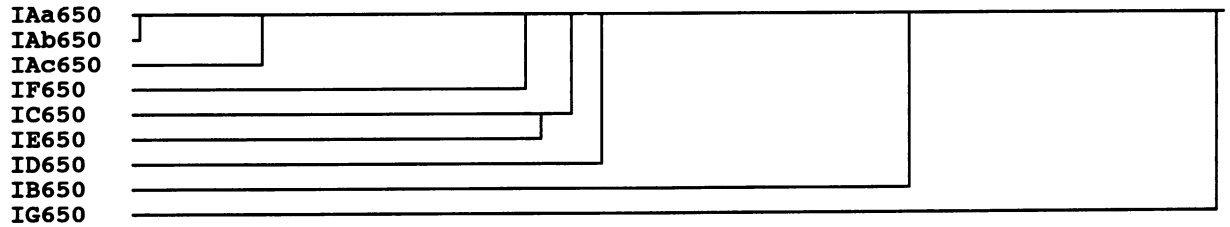

FIG. 4. Dendrogram comparing primary amino acid sequences of the known toxin gene classes. The MULTALIN software (9) was used to align amino acids 1 to 650 of all known CryI toxin subclasses and group them into hierarchical clusters. References of the utilized sequences were CryIA(a) (34), CryIA(b) (13), CryIA(c) (2), CryIB (6), CryIC (29), CryID (17), CryIE (4), CryIF (8), and CryIG (13a).

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