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Properties of a 72-Kilodalton Mosquitocidal Protein from *Bacillus thuringiensis* subsp. *morrisoni* PG-14 Expressed in *B. thuringiensis* subsp. *kurstaki* by Using the Shuttle Vector pHT3101

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The mosquitocidal properties of *Bacillus thuringiensis* subsp. *israelensis* and *B. thuringiensis* subsp. *morrisoni* PG-14 are attributable to protein inclusions grouped together within a parasporal body. In both of these strains, the mosquitocidal activity resides in proteins with molecular masses of 27, 72, 128, and 135 kDa. In an attempt to determine the toxicity of each protein, the shuttle vector pHT3101 was used to express the *cryIVD* gene (encoding the 72-kDa CryIVD protein) from *B. thuringiensis* subsp. *morrisoni* in an acrystalliferous mutant of *B. thuringiensis* subsp. *kurstaki*. With this system, parasporal inclusions of the 72-kDa protein were obtained that were comparable in size, shape, and toxicity to those produced by parental *B. thuringiensis* subsp. *morrisoni*. The inclusions were bar shaped, measured 500 by 300 by 150 nm, and were easily visible with phase-contrast microscopy by 16 h of cell growth. A 50% lethal concentration of 64 ng/ml for these inclusions was determined in bioassays against fourth instars of *Culex quinquefasciatus*, which was similar to the 50% lethal concentration of 55 ng/ml obtained for the 72-kDa inclusion from *B. thuringiensis* subsp. *israelensis*. In contrast, expression of the *cryIVD* gene in *Escherichia coli* was very low and only detectable by immunoblot analysis. These results demonstrate that the pHT3101-*B. thuringiensis* expression system can be used to express the CryIVD protein in quantities and with properties comparable to that obtained with the natural host. This system may prove useful for the expression of other *B. thuringiensis* proteins and, in particular, for reconstitution experiments with inclusions produced by the mosquitocidal subspecies of *B. thuringiensis*.

Bacillus thuringiensis subsp. *israelensis*, a highly insecticidal gram-positive bacterium, is used in the United States and Africa to control larvae of mosquitoes and blackflies, vectors of important human diseases such as malaria and filariasis (15, 25). *B. thuringiensis* subsp. *israelensis* was discovered in Israel in 1976, but, more recently, another bacterium with a similar spectrum of insecticidal activity, the PG-14 isolate of *B. thuringiensis* subsp. *morrisoni*, was isolated in the Philippines (26). The toxicity of both of these bacterial isolates is due to the presence of proteins with molecular masses of 28, 72, 125, and 135 kDa (8–12).

The contribution that each protein makes to the toxicity of the parasporal body is not well understood, but it appears that two or more proteins are required and possibly these proteins act synergistically to yield full toxicity (4, 19, 34, 35). Data in most of these studies indicate that the 27-kDa protein is responsible for enhancing the toxicity of the other proteins, but synergistic interactions between the 128- and 135-kDa proteins have also been reported (5). A recent study, however, suggests that the 27-kDa protein contributes little, if any, to the toxicity of the intact parasporal inclusion (6).

A major problem in defining the toxicity of individual parasporal body proteins is that, in most studies, the proteins were purified biochemically and then assayed either in a soluble form or after precipitation (3, 4, 20, 22, 34). The level of homogeneity for individual proteins, therefore, remains uncertain, and, furthermore, precipitation of the proteins does not yield inclusions representative of wild-type bacteria. This could be important in the accurate determination of toxicity since bioassays depend on the ingestion of the proteins in particulate form from suspensions by the filter-feeding mosquito or blackfly larva. On the other hand, where the genes encoding parasporal body proteins have been cloned and expressed in alternative bacterial hosts such as *Escherichia coli* (2, 24, 31–33), *Bacillus subtilis* (33), or *Bacillus megaterium* (7), the levels of expression were variable, and either the proteins remained in a soluble form or the inclusions produced were not characteristic of those formed by natural hosts (2, 7, 16, 24, 31).

To determine whether the problems encountered with purity and physical consistency could be overcome by using *B. thuringiensis* as an expression host for cloned genes, the *cryIVD* gene (18) encoding a 72-kDa mosquitocidal protein from *B. thuringiensis* subsp. *morrisoni* PG-14 was cloned and its expression in *E. coli* and an acrystalliferous mutant of *B. thuringiensis* subsp. *kurstaki* was compared by using the *E. coli-B. thuringiensis* shuttle vector pHT3101 (23). The CryIVD protein was selected because, when it is produced naturally by wild-type subspecies, this protein forms bar-shaped inclusions that can be isolated from the parasporal body, providing a useful control for assessing the properties of proteins and inclusions produced in our experiments. Additionally, recent studies have shown that the sequences of the *cryIVD* genes of *B. thuringiensis* subsp. *morrisoni* and *B. thuringiensis* subsp. *israelensis* are virtually identical (11), making the results obtained with the gene from the former species relevant to the latter.

In this article, we report the results of our expression studies which show that high levels of expression yielding inclusions comparable to those produced by the natural host can be obtained by using the shuttle vector pHT3101 and *B. thuringiensis* as an expression system.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and general methods. The bacterial strains used in this study were *E. coli* JM101 and an acrystalliferous mutant (Cry*B*), *B. thuringiensis* subsp. *kurstaki* HD1, which was obtained from A. Aronson, Department of Biology, Purdue University. The plasmid pM1, which contains the *cryIVD* gene encoding the 72-kDa mosquitocidal protein of *B. thuringiensis* subsp. *morrisoni* isolate PG-14, has been described (12). The shuttle vector pHT3101 (23), containing origins of replication in both *B. thuringiensis* and *E. coli*, has Amp^r and Em^r as selectable markers and a multiple cloning site in the *lacZ* gene. The vector was obtained from D. Lereclus, Institut Pasteur, Paris. Restriction enzyme digestion, ligation, and transformation in *E. coli* were performed by using standard protocols (28).

Construction of the plasmids pCG1 and pCG7. The plasmid pM1 was digested with *Pst*I, and the 5.6-kb fragment, which contains the *cryIVD* gene, an associated gene encoding a 20-kDa protein (1) and a truncated *cytA* gene (encoding the 27-kDa protein), was separated on agarose and purified by Geneclean (Bio 101, La Jolla, Calif.). The purified fragment was ligated into a unique *Pst*I site of pHT3101 and then used for transformation in *E. coli* JM101. Transformants, which were selected on Luria-Bertani (LB) agar containing ampicillin (50 μg/ml), erythromycin (25 μg/ml), 20 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (80 μg/ml), were isolated, and the size and orientation of the *Pst*I inserts were determined. Two transformants, pCG1 and pCG7, were isolated. Both were 12.2 kb but contained the *cryIVD* gene in opposite orientations to the *lacZ* promoter (see Fig. 1).

Transformation of *B. thuringiensis*. *B. thuringiensis* cells were transformed by electroporation essentially as described previously (29). Briefly, *B. thuringiensis* subsp. *kurstaki* HD1 Cry*B* cells were grown with shaking at 37°C in 100 ml of LB medium to an optical density at 600 nm of 0.2. The cells were washed once in 100 ml of 4°C water and resuspended in 1.6 ml of cold electroporation buffer (400 mM sucrose, 1 mM $MgCl_2$, 7 mM $NaKHPO_4$ [pH 6.0]) to a concentration of ca. 10^9 cells per ml. Aliquots (400 μl) of this cell suspension were mixed with 0.1 μg of plasmid DNA (pCG1 or pCG7) in 0.2-cm electroporation cuvettes (Bio-Rad, Richmond, Calif.) and kept on ice for 10 min. Electroporation was performed in a Bio-Rad Gene Pulser apparatus, which was set at 25 μF and 1.3kV, and a Bio-Rad Pulse Controller set at 200 Ω. The cuvettes were placed in a safety chamber, and the pulse was applied once. After electroporation, the cells were kept on ice again for 10 min. A 1-ml volume of LB medium was then added, and the cells were incubated with shaking at 37°C for 1 h. The cells were then plated on nutrient agar plates containing 50 μg of ampicillin and 25 μg of erythromycin, each per ml. Colonies were analyzed by minipreps for the presence of pCG1 or pCG7.

Expression of the *cryIVD* gene. The *E. coli* JM101 and *B. thuringiensis* subsp. *kurstaki* strains containing the plasmids pCG1 and pCG7 were analyzed for expression of the *cryIVD* gene by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. For the *E. coli* strains, overnight cultures with or without 1 mM IPTG induction were used for expression analyses. The *B. thuringiensis* subsp. *kurstaki* isolates CG1-1 and CG1-2 (transformed with pCG1) and CG7-1 (transformed with pCG7) were grown on nutrient agar plates containing 50 μg of erythromycin per ml for 5 days at 30°C to ensure sporulation and complete cell lysis. The bacterial lysates were isolated by washing the plates with deionized water and sedimented by centrifugation at 15,000 × *g* for 10 min. The pellets were boiled with sample treatment buffer and analyzed by discontinuous SDS-PAGE (21) by using 4.5% acrylamide (pH 6.8) and 10% acrylamide (pH 8.8) as the stacking and separating gels, respectively. After electrophoresis, the gels were stained with 0.1% Coomassie blue R-250.

For immunoblotting, the proteins resolved by SDS-PAGE were transferred to nitrocellulose for 16 h at a constant current of 250 mA. The nitrocellulose was then probed with rabbit antibody developed against either the purified intact parasporal inclusion or the purified 72-kDa protein of *B. thuringiensis* subsp. *israelensis* by using methods previously described (14). Goat anti-rabbit immunoglobulin G–alkaline phosphatase was used as the second antibody, and chromogenic development was then achieved with nitroblue tetrazolium chloride (1 mg/ml in H_2O) and 5-bromo-4-chloro-3-indolyl phosphate (5 mg/ml in dimethylformamide).

Purification of the 72-kDa parasporal inclusion. *B. thuringiensis* subsp. *kurstaki* CG1-1 was cultured for 5 days on nutrient agar plates as described above. The cells, most of which had lysed, and the parasporal inclusions were washed from the plate, sedimented as described above, and washed once with 50 mM NaBr–10 mM ETDA. The pellet was resuspended in water and sonicated (duty cycle, 50%; output control, setting 5; Branson Sonifier) for 5 min on ice, and then it was subjected to centrifugation at 20,000 rpm for 1 h in an SW28 rotor by using a discontinuous NaBr gradient of 38.5% (4 ml), 41.9% (6 ml), 45.3% (6 ml), 48.9% (6 ml), 52.7% (6 ml), and 56.3% (3 ml) essentially as previously described (19). The partially purified 72-kDa parasporal inclusions were then recentrifuged in another discontinuous NaBr gradient of 38.5% (5 ml), 40% (7 ml), 42% (7 ml), 44% (6 ml), and 47% (1.5 ml) under the same centrifugation conditions. The purified 72-kDa parasporal inclusions were collected, washed several times with cold, deionized water to remove all of the NaBr, and then resuspended in 10 mM ETDA and stored at 4°C until needed. Intact parasporal inclusion bodies from the wild-type *B. thuringiensis* subsp. *morrisoni* grown on GYS broth were purified as previously described (19).

Electron microscopy. Purified 72-kDa inclusions or the intact parasporal bodies of *B. thuringiensis* subsp. *morrisoni* were sedimented by centrifugation in a 1.5-ml Eppendorf tube. The resulting pellet was dislodged from the tube bottom, suspended in 250 μl of agar, and fixed for 2 h in 3% glutaraldehyde (in 0.1 M cacodylate buffer [pH 7.4]). After postfixation in 1% OsO_4 (in the same buffer), the pellet was dehydrated in an ethanol-propylene oxide series and embedded in Epon-Araldite. Ultrathin sections were cut on a Sorvall MT-5000 microtome, stained with uranyl acetate and lead citrate, and examined in a Hitachi 600 electron microscope operating at an accelerating voltage of 50 to 75 kV. Intact parasporal bodies of *B. thuringiensis* subsp. *morrisoni* were prepared in the same manner.

Bioassays. The toxicity of each of the transformants was determined against mosquito larvae. For *E. coli* strains harboring either pCG1 or pCG7, 0.2 ml of an overnight culture (5×10^{10} cells per ml) was added to 1.8 ml of distilled water containing 10 second-instar *Culex quinquefasciatus* larvae. The mortality was observed at 24 h by counting the number of surviving larvae. Bioassays were performed in triplicate.

The toxicity of purified 72-kDa inclusions from *B. thuringiensis* subsp. *kurstaki* strains containing either pCG1 or

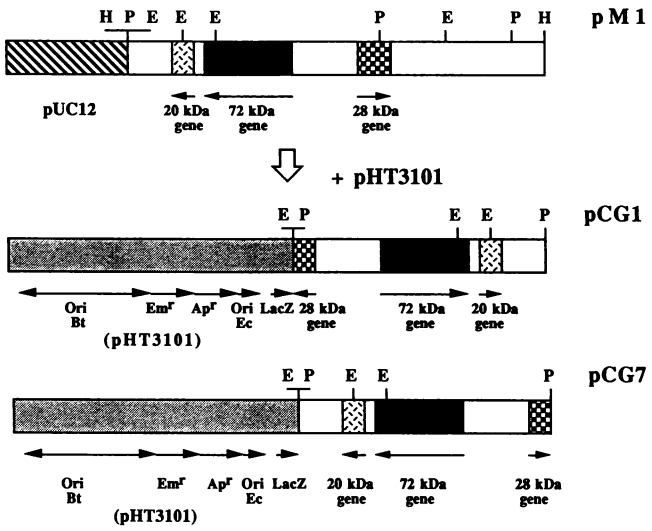

FIG. 1. Plasmid maps for the cloning of the 72-kDa protein from *B. thuringiensis* subsp. *morrisoni* into the shuttle vector pHT3101. The 5.6-kb *Pst*I fragment of pM1, derived from *B. thuringiensis* subsp. *morrisoni* PG-14 and containing the *cryIVD* and the 20-kDa protein genes, was isolated and ligated into the *Pst*I site of pHT3101. Both orientations of the expected plasmid were isolated. The plasmids pCG1 and pCG7 have the *Pst*I fragment in the same and opposite orientations relative to the *lacZ* gene, respectively.

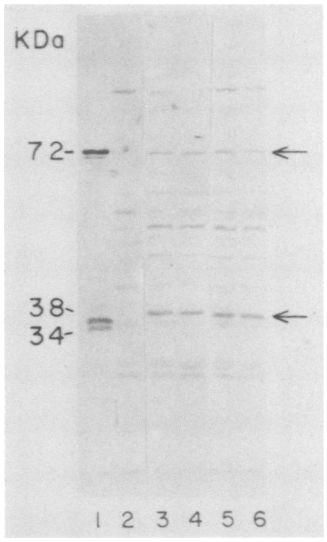

FIG. 2. Detection of the *B. thuringiensis* subsp. *morrisoni* 72-kDa protein gene expression products in *E. coli* by immunoblotting. Rabbit antiserum developed against the 72-kDa protein of *B. thuringiensis* subsp. *israelensis* was used for detection. Lanes: 1, pure 72-kDa inclusion of *B. thuringiensis* subsp. *israelensis*; 2, pHT3101; 3, pCG7 (IPTG induced); 4, pCG7; 5, pCG1 (IPTG induced); 6, pCG1. Lane 1 was loaded with 50 ng of purified 72-kDa parasporal inclusions, and lanes 2 to 6 were loaded with 40 μl of crude overnight bacterial culture. The arrows indicate the products of the 72-kDa protein gene.

pCG7 was assayed against fourth-instar *C. quinquefasciatus* larvae. Briefly, 20 larvae were placed in 99.9 ml of distilled water to which 0.1 ml of the parasporal inclusions was added. To obtain a 50% lethal concentration (LC_{50}), seven different concentrations (20 to 800 ng/ml) of inclusions were used, with four replicates per concentration. Mortality was observed at 24 h, and LC_{50}s were calculated by probit analyses by using a Basic program (27). For comparison, the LC_{50} of the wild-type, purified 72-kDa parasporal inclusion from *B. thuringiensis* subsp. *israelensis* was determined as described above. Controls consisted of larvae reared similarly but not exposed to the bacterial toxin inclusions.

RESULTS

Constructs and analyses of *cryIVD* gene expression. Two plasmid constructs, pCG1 and pCG7, harboring the *cryIVD* gene of *B. thuringiensis* subsp. *morrisoni* PG-14 in opposite orientations to the *lacZ* gene, are illustrated in Fig. 1. *E. coli* transformed with pCG1 or pCG7 expressed the 72-kDa protein at very low levels such that expression was detectable only by immunoblotting (Fig. 2, lanes 4 and 6). The expression of the *cryIVD* gene in *E. coli* was not inducible by IPTG (Fig. 2, lanes 3 and 5). Furthermore, the 72-kDa protein that was expressed was considerably less stable, resulting in the formation of the 34- to 40-kDa proteolytic fragments (Fig. 2, lanes 3 to 6). Control *E. coli* harboring only the pHT3101 vector did not produce any detectable protein of 72 kDa (Fig. 2, lane 2).

No toxicity was observed when larvae of *C. quinquefasciatus* were exposed for 24 h to *E. coli* lysates from either of the *E. coli* strains expressing the *cryIVD* gene. Control *E. coli* harboring only the pHT3101 vector also displayed no mosquito toxicity.

In contrast to the results obtained with *E. coli*, the expression of the *cryIVD* gene was significantly higher in *B. thuringiensis* subsp. *kurstaki*. Strains CG1-1 and CG1-2, transformed with pCG1, and CG7-1, transformed with pCG7, all expressed the 72-kDa protein at approximately the same level and at a level readily detectable by SDS-PAGE (Fig. 3, lanes 1 to 3). The expression of the 72-kDa toxin is independent of the orientation of the *Pst*I fragment (Fig. 3,

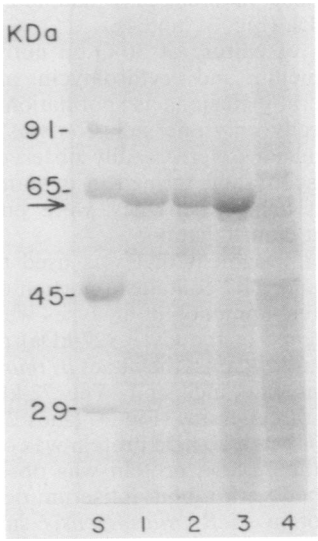

FIG. 3. SDS-PAGE analysis of the expression of the 72-kDa gene in *B. thuringiensis* subsp. *kurstaki* Cry*B*. Lanes: 1, clone CG1-1; 2, clone CG1-2; 3, clone CG7-1; 4, Cry*B*; S, standard proteins. Lanes 1 to 4 were each loaded with 40 μl of crude overnight bacterial culture. The arrow indicates the 72-kDa protein.

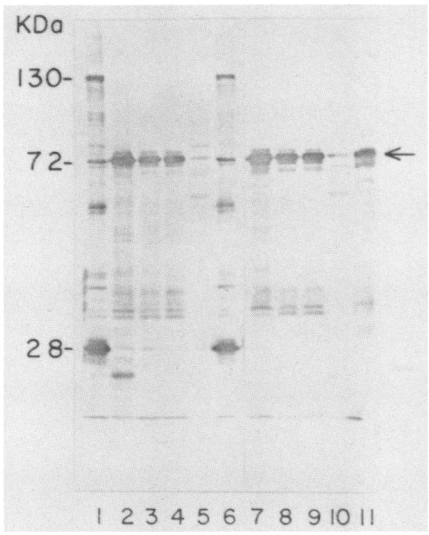

FIG. 4. Immunoblot analysis of the expression products of the *B. thuringiensis* subsp. *morrisoni* 72-kDa protein gene in *B. thuringiensis* subsp. *kurstaki* Cry*B*. Lanes 1 to 6 were probed with rabbit antiserum developed against the intact whole parasporal inclusion of *B. thuringiensis* subsp. *israelensis*, while lanes 7 to 11 were probed with rabbit antiserum developed against the 72-kDa protein of *B. thuringiensis* subsp. *israelensis*. Lanes: 1 and 6, intact whole parasporal inclusion of *B. thuringiensis* subsp. *israelensis* (500 ng); 2 and 7, clone CG1-1; 3 and 8, clone CG1-2; 4 and 9, clone CG7-1; 5 and 10, Cry*B* with pHT3101; 11, purified 72-kDa parasporal inclusion from *B. thuringiensis* subsp. *israelensis*. Lanes 2 to 5 and 7 to 10 were each loaded with 2 μl of an overnight bacterial culture. The arrow indicates the 72-kDa protein.

lanes 1 and 3). Furthermore, the SDS-PAGE profile of the cloned 72-kDa protein of *B. thuringiensis* subsp. *morrisoni* PG-14 is similar to the profile of the NaBr gradient-purified 72-kDa protein of *B. thuringiensis* subsp. *israelensis*. *B. thuringiensis* subsp. *kurstaki* Cry*B* had no major observable protein of 72 kDa (Fig. 3, lane 4).

After 5 days of culture at 30°C on nutrient agar plates containing ampicillin and erythromycin, all three isolates formed parasporal inclusions as sporulation neared completion, and generally only one inclusion was formed in each cell. These inclusions were readily observed under phase-contrast microscopy and formed in quantities sufficient to form a distinct band when they were purified by NaBr density gradient centrifugation.

Since the *Pst*I fragment that was used to construct the plasmids CG1 and CG7 contains a portion of the *cytA* gene with its promoter, immunoblot analyses were performed to detect the presence of the CytA (27-kDa) protein by using antibodies prepared against the intact *B. thuringiensis* subsp. *israelensis* parasporal inclusion. The 72-kDa protein was observed in all three strains (Fig. 4, lanes 2 to 4); however, in one strain, CG1-1, a 20-kDa protein was observed (Fig. 4, lane 2). Only the 72-kDa protein was observed when the blots were probed with rabbit antiserum developed against the 72-kDa protein of *B. thuringiensis* subsp. *israelensis* (Fig. 4, lanes 7 to 9). None of the 20-, 27-, and 72-kDa proteins were detected in *B. thuringiensis* subsp. *kurstaki* (Fig. 4, lanes 5 and 10).

Inclusion size and shape. Analyses of the recombinant parasporal inclusions by electron microscopy showed that they are similar, if not identical, in shape to the 72-kDa parasporal inclusions formed in wild-type *B. thuringiensis* subsp. *morrisoni* isolate PG-14 and are slightly larger in size (Fig. 5). The inclusions were bar shaped and variable in size but were in the range of 500 by 300 by 150 nm.

TABLE 1. Toxicity of purified and cloned mosquitocidal proteins to fourth-instar larvae of *C. quinquefasciatus*

Protein	LC_{50} (ng/ml)	LC_{95} (ng/ml)
B. thuringiensis subsp. *israelensis*		
Whole crystal	3.8 (3.5–4.2)[a]	15.5 (13.4–18.7)[a]
72-kDa inclusion, purified	55 (45–67)	194 (133–290)
B. thuringiensis subsp. *kurstaki*, Cry*B*, isolate CG1-1		
Crude bacterial culture	549 (342–1,092)	>10,000
Cloned 72-kDa inclusion, purified	64 (48–84)	247 (140–443)

[a] Values in parentheses indicate fiducial limits. Confidence level, 95%.

Toxicity. Both the crude bacterial culture and parasporal inclusions purified from the CG1-1 strain were highly toxic to fourth-instar *C. quinquefasciatus* larvae, with LC_{50}s of ca. 549 and 64 ng/ml, respectively (Table 1). In comparison, the LC_{50} for purified 72-kDa inclusions from *B. thuringiensis* subsp. *israelensis* was 55 ng/ml. The LC_{50} of the intact parasporal inclusion of *B. thuringiensis* subsp. *israelensis* was 3.84 ng/ml when assayed against the same stage and species.

DISCUSSION

The high levels of the 72-kDa protein obtained with pHT3101 in *B. thuringiensis* clearly demonstrate the applicability of using a closely related host and suitable vector as an expression system. Not only was the level of expression with this system comparable to that obtained with *B. thuringiensis* subsp. *morrisoni*, the host from which the gene was cloned, but the size and shape of the inclusions were typical of those produced by this bacterium (Fig. 5) (19, 20). This is an interesting finding because, in the parasporal body produced by *B. thuringiensis* subsp. *morrisoni*, the 72-kDa bar-shaped inclusion occurs along with the other inclusions which contain the 27-, 128-, and 135-kDa proteins. Moreover, each of these inclusions is surrounded by a multilaminar envelope. It is possible that the occurrence of the other inclusions, all of which are apparently formed simultaneously with the 72-kDa inclusion, and the presence of the envelope play some role in the size and shape of the inclusions formed by the wild-type host. For example, one might expect larger inclusions or a greater number of them to occur in the recombinant *B. thuringiensis* subsp. *kurstaki* in the absence of any of the other proteins; however, neither occurs to any significant extent. The results obtained in the present study, therefore, indicate that the absolute quantity of the 72-kDa protein produced and its crystallization to form a bar-shaped inclusion with more-or-less uniform dimensions are probably inherent properties of the *cryIVD* gene and CryIVD protein.

It is possible that the 20-kDa product of a gene (1), located 289 bp downstream from the *cryIVD* gene, could contribute to the high levels of expression observed in *B. thuringiensis* subsp. *kurstaki* because this gene also occurs on the 5.6-kb

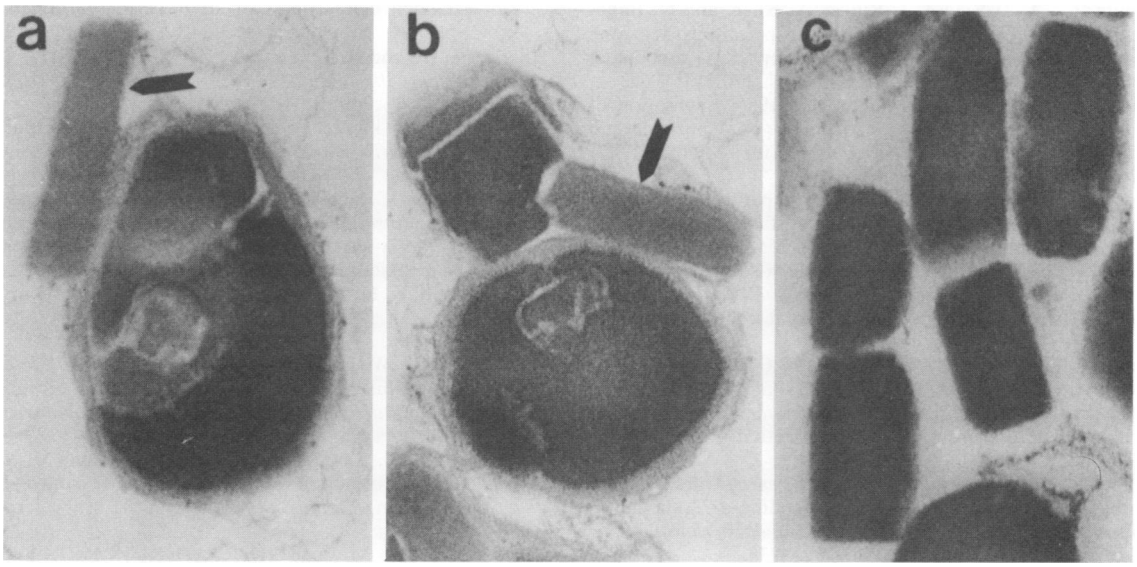

FIG. 5. Electron micrographs of purified parasporal bodies from naturally occurring and recombinant strains of *B. thuringiensis*. (a and b) Parasporal bodies of the PG-14 isolate of *B. thuringiensis* subsp. *morrisoni* illustrating the characteristic bar-shaped inclusion (arrows) which is composed of the 72-kDa CryIVD protein. (c) Parasporal bodies from *B. thuringiensis* subsp. *kurstaki* transformed with the shuttle expression vector pHT3101 carrying the *cryIVD* gene. Magnification, ×29,700.

*Pst*I fragment that was used in transformation. In *E. coli*, expression of the gene encoding the 20-kDa protein is essential for the expression and stability of the 27-kDa protein of *B. thuringiensis* subsp. *israelensis* because of its protective role against proteolytic cleavage of the 27-kDa protein or alternatively by providing a matrix for crystallization of the 27-kDa protein (30).

A similar mechanism of stabilization of the 72-kDa protein by the 20-kDa protein could occur in *B. thuringiensis* since it is synthesized prior to the synthesis of the 72-kDa protein (data not shown). It has been proposed (1) that genes encoding the 72-kDa and 20-kDa proteins are part of the same transcriptional unit. While we have no strong evidence to dispute this claim, the 20-kDa protein is translated before the 72-kDa protein.

In *E. coli*, the 20-kDa protein does not appear to play a significant role in the production of the 72-kDa protein. Only low levels of expression of the *cryIVD* gene in *E. coli* were observed. Furthermore, the 72-kDa protein was readily proteolyzed to 34- to 40-kDa fragments (Fig. 2), suggesting that the 20-kDa protein does not cause any stabilization of the 72-kDa protein as was observed with the 27-kDa protein (30). This low expression level is probably due to two factors, poor recognition of the *cryIVD* gene promoter by the *E. coli* polymerase(s) and proteolytic cleavage of the 72-kDa protein soon after it is synthesized. Furthermore, it appears that the low level of expression in *E. coli* results because the polymerase(s) apparently uses the *B. thuringiensis* promoter rather than the *lacZ* promoter as evidenced by a lack in induction of expression by IPTG.

The similar LC_{50}s obtained for the 72-kDa inclusions produced naturally by *B. thuringiensis* subsp. *israelensis* or *B. thuringiensis* subsp. *kurstaki* by using pHT3101 (Table 1) show that the inclusions isolated from the wild-type parasporal bodies can be relatively pure. However, this example cannot be extended to other inclusions in the parasporal body of *B. thuringiensis* subsp. *morrisoni* and *B. thuringiensis* subsp. *israelensis* because these inclusions are much more tightly bound in the parasporal body by the envelope than is the 72-kDa inclusion (19, 20). Thus, the prudent way to determine the toxicity of the other parasporal body proteins would be to express each cloned gene individually, and, on the basis of our results, pHT3101 and the acrystalliferous *B. thuringiensis* subsp. *kurstaki* used here provide a good expression system for this purpose. If results obtained with the other genes are comparable to those obtained with the *cryIVD* gene, the products can be used for reconstitution experiments aimed at determining the contribution that individual proteins make to the toxicity of the parasporal body of mosquitocidal subspecies of *B. thuringiensis*.

Although an acrystalliferous strain of *B. thuringiensis* subsp. *kurstaki* was used in this study, it is likely that other subspecies of *B. thuringiensis* that can be transformed should also work well as expression hosts for genes inserted in pHT3101. In the original description of pHT3101 (23), the *cryIA(a)* gene was expressed by using *B. thuringiensis* subsp. *kurstaki* Cry*B*. High levels of expression were obtained, but the size and shape of the inclusions produced, if any, were not determined. The resemblance of the 72-kDa inclusions produced by using pHT3101 to those produced by the natural hosts suggests that this system may also be useful for producing other *B. thuringiensis* proteins rather than using hosts such as *E. coli* (2, 13, 17, 24, 31–33), *B. subtilis* (31, 33), and *B. megaterium* (7).

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