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# Structural Relatedness between Mosquitocidal Endotoxins of *Bacillus thuringiensis* subsp. *israelensis*

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A mosquitocidal toxin gene, cloned from *Bacillus thuringiensis* subsp. *israelensis*, was introduced into mutant crystal-negative *B. thuringiensis* subsp. *israelensis* cells. Partial toxicity to mosquitos was restored. The 58-kilodalton cloned gene product is a minor protein component of *B. thuringiensis* subsp. *israelensis* crystals and is structurally related to a major, 135-kilodalton crystal toxin.

Insecticidal proteins are synthesized during sporulation of many subspecies of *Bacillus thuringiensis*. The insecticidal proteins accumulate intracellularly as parasporal crystals. For *B. thuringiensis* subsp. *israelensis*, the toxic proteins are lethal to the larval stages of mosquitos (3) and blackflies (13). Control of these dipteran species is important mainly because they are vectors for human diseases such as malaria and onchocerciasis (6). For *B. thuringiensis* subsp. *israelensis*, the toxic crystals are composed of four major protein species with molecular sizes of about 135, 70, 38, and 28 kilodaltons (kDa) (12). There are some minor species as well. These sizes reflect consensus values, taking into consideration several subsequent confirmatory published reports. Pfannenstiel et al. (9) tested the immunological relatedness between certain of these crystal proteins by reactivity with monospecific polyclonal antiserum. Although the 135-, 70-, and 28-kDa proteins are immunologically distinct, the 38-kDa species is derived from the 70-kDa protein by proteolysis (9). Thus, there appears to be three separate gene products constituting most of the crystal mass.

In our earlier reports (11, 14) we described the cloning of a gene from *B. thuringiensis* subsp. *israelensis* that coded for a protein in *Escherichia coli* or *Bacillus subtilis* of about 58-kDa that was mosquitocidal to larvae of *Aedes aegypti*. We expressed the gene in vitro and could detect products up to about 72 kDa, matching the size of the open reading frame present in the DNA sequence. We proposed that the primary gene product was proteolytically processed in vivo. At the time, we did not rule out the possibility that the cloned 58-kDa-coding gene coded for the 70-kDa major crystal protein. In addition, we noted the surprising sequence similarity between the 58-kDa toxin gene and a 130-kDa toxin gene with lepidopteran specificity that we had cloned from *B. thuringiensis* subsp. *kurstaki* (11). In the present report, we show that the product of the 58-kDa cloned gene is also accumulated in *B. thuringiensis* subsp. *israelensis* as a 58-kDa species, that this toxin is a minor component of the crystals, and that it is structurally related to the large 135-kDa crystal protein.

To introduce the cloned 58-kDa gene back into *B. thuringiensis* subsp. *israelensis*, we developed a plasmid transformation protocol on the basis of the method of Chang and Cohen (2). First, a spontaneous crystal-negative derivative of our wild-type *B. thuringiensis* subsp. *israelensis* strain (ONR60A, from J. Hoch) was obtained after the chance observation that the isolate had lost its large plasmid. We

found that this crystal-negative mutant had lost the large plasmid associated with the production of parasporal crystals toxic to diptera (14). This was the same plasmid from which we cloned the 58-kDa toxin gene into *E. coli* and *B. subtilis* (14). Protoplasts were prepared of the crystal-negative mutant and its wild-type parent by the method of Chang and Cohen (2) with two modifications. Cells were treated with a solution containing lysozyme (10 mg/ml), sucrose (25% [wt/vol]), 50 mM Tris hydrochloride (pH 7.5), and 20 mM EDTA, until about 98% was converted to protoplasts (usually 2 to 4 h), after which 5 μg of pSY408 was added. After transformation, the cells were regenerated by shaking for 24 h in DM3 medium at 30°C, washed and then suspended in Luria broth with kanamycin (between 10 and 50 μg/ml) for 24 h, followed by selection for growth on plates containing the same medium with kanamycin (25 μg/ml). The amounts of antibiotic must be adjusted for the background resistance of the recipient strain. Plasmid pSY408 carries the structural gene and flanking DNA sequences for the 58-kDa polypeptide (a total of about 4,500 base pairs) inserted in vector pUB110 at the *Eco*RI restriction site (11). The vector pUB110 arose in *Staphylococcus aureus* but replicates also in *B. subtilis* and *B. thuringiensis*. The plasmid carries a gene conferring resistance to kanamycin, and the entire sequence has been determined (7, 8). Two transformants of each recipient were chosen for further study. They were resistant to kanamycin and were shown to carry an intact pSY408 plasmid. Cell cultures were grown to late stationary phase or about 8 h after the end of log-phase growth, and then equivalent samples (cell number) were placed in microtiter wells with six or more second- and third-instar mosquito larvae (*A. aegypti*). Wild-type *B. thuringiensis* subsp. *israelensis* (with or without the plasmid pSY408) killed all the feeding larvae within 6 h, whereas the crystal-negative derivative carrying pSY408 killed all the larvae in 18 h. One-half maximal killing was observed for wild type at 90 min and for *cry⁻* plus pSY408 at 12 h. The crystal-negative strain alone has no lethal effect over 30 h of monitoring, the end of the assay.

In preparation for electrophoresis, samples of equivalent numbers of stationary-phase bacterial cells were collected by centrifugation and suspended in a mixture of 62.5 mM Tris hydrochloride (pH 6.8), 1% (wt/vol) sodium dodecyl sulfate, 0.1% (vol/vol) mercaptoethanol, 10% (vol/vol) glycerol, and bromophenol blue. They were heated in a boiling water bath for 4 min immediately before electorphoresis. Samples of protein from each transformant were separated by electrophoresis through denaturing sodium dodecyl sul-
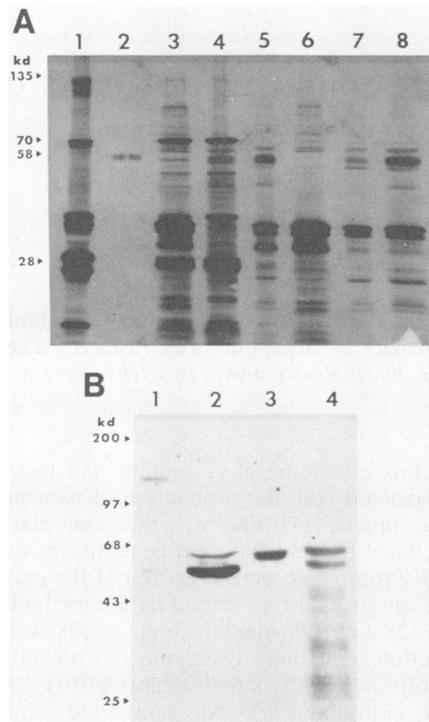
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FIG. 1. Electorphoretic separation of proteins from crystals and cells of *B. thuringiensis* subsp. *israelensis* carrying the cloned 58-kDa toxin gene. Proteins were transferred to a nitrocellulose filter and reacted with polyclonal rabbit antiserum raised against whole crystals (A) or antibodies eluted after binding to a single 58-kDa protein band (B). The samples for both panels were from *B. thuringiensis* subsp. *israelensis*, with the noted exception: whole-crystal proteins (lane 1); partially pure 58-kDa cloned product from *B. subtilis* (lane 2); wild-type total cell protein (lane 3); proteins from two isolates of wild type transformed with pSY408 (lanes 4 and 5); crystal-negative total cell protein (lane 6); and proteins from two isolates of crystal-negative cells transformed with pSY408 (lanes 7 and 8). The sizes and positions of *B. thuringiensis* subsp. *israelensis* crystal proteins (A) and molecular weight standards (B) are indicated at the left. kd, Kilodaltons.

fate polyacrylamide gels (4), by using a gradient of 8 to 15% (wt/vol) acrylamide. The separated proteins were then transferred to nitrocellulose and treated with polyclonal antiserum raised in rabbits against whole-crystal preparations from *B. thuringiensis* subsp. *israelensis* (11, 14). The bound antibodies were visualized by reaction with [$^{125}$I]protein A and autoradiography. Figure 1, panel A, is a photograph of the X-ray film exposed to the immunoblot. A sample of semipurified crystal proteins and 58-kDa cloned product are included in lanes 1 and 2, respectively. A comparison of the seroreactive proteins of lanes 3 to 8 showed that crystal-negative and wild-type *B. thuringiensis* subsp. *israelensis* cells transformed with plasmid pSY408 accumulated the 58-kDa cloned gene product. More of the toxin was observed in stationary phase than during log-phase growth (not shown). During the transformation process one of the wild-type recipients (lane 5) lost its large plasmid and failed to accumulate the crystal proteins of 135, 70, and 28 kDa. Therefore, the main product of our cloned toxin gene is 58 kDa, even when expressed in its natural host, and so it is unlikely that this gene is the same as that which codes for the 70-kDa crystal protein. We believe that the primary transla-

tion product of the 58-kDa gene is probably 72 kDa, on the basis of the size of the sequenced open reading frame and the products observed after in vitro translation (11). We think that this is rapidly processed by proteolysis to the accumulated 58-kDa size.

Using unfractionated polyclonal rabbit antiserum, we were not able to detect the 58-kDa product in preparations of crystal proteins that were immunoblotted from denaturing polyacrylamide gels. To circumvent this problem, we eluted (5) antibodies from the single 58-kDa gel band on immunoblots of proteins expressed in *B. subtilis* (9). As before, protein samples were separated by electorphoresis and immunoblotted with the antigen-selected antibody preparation. Panel B of Fig. 1 shows the results. The antigen-selected antibodies reacted with a species in the crystal protein preparation of 58 kDa (lane 1), matching the major reactive band in the partially purified cloned product (lane 2). However, in the whole-cell extract from wild-type *B. thuringiensis* subsp. *israelensis* (lane 3) we still could not detect the 58-kDa gene product. We were surprised to find that the antigen-selected antibodies reacted not only with the low-abundance 58-kDa protein in the crystal preparations, but also with the high-abundance 135-kDa species. No reaction was seen to the other major crystal proteins at 70 or 28 kDa. This suggests a structural relatedness between the 135- and 58-kDa proteins. A cross-reacting band at about 64 kDa was present in all the extracts from either *B. subtilis* or *B. thuringiensis* subsp. *israelensis*, whether or not pSY408 was present (gel not shown).

We compared the sequence of the cloned 28-kDa gene (15, 16) with our 58-kDa gene (11) and found no similarities. We earlier reported that in contrast to the 28-kDa protein, the 58-kDa protein showed no hemolytic activity (11). However, we did find a sequence relatedness to the 130-kDa lepidopteran toxin from *B. thuringiensis* subsp. *kurstaki*. Those results, coupled with our present findings, point out the possible similarities between the larger of the crystal proteins from these two subspecies. Whether the relatedness reflects common structure, as for example in protein alignment during formation of the crystal, or common function, as in physiological mode of toxin action, is not yet known.

The gene for the 135-kDa toxin has also been cloned and expressed (10). It is interesting that a more recent isolation of that same gene (1) shows that it is separated physically from our 58-kDa toxin gene by as few as 2,000 base pairs on the 72-MDa plasmid in *B. thuringiensis* subsp. *israelensis*. Perhaps the larger gene is a progenitor in an evolutionary sense to the smaller and preserves some structural features that are recognizable with antibodies. A direct comparison will be possible when the DNA of the large gene is sequenced. The reason why *B. thuringiensis* subspp. *israelensis* and *kurstaki* each maintain multiple toxin genes remains open to speculation. However, by applying the gene transformation methods described in this paper, it will be possible to study the contributions to toxicity from the distinct gene products that constitute the mosquitocidal crystals of *B. thuringiensis* subsp. *israelensis*.

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