JOURNAL OF BACTERIOLOGY, May 1990, p. 2217–2223
0021-9193/90/052217-07$02.00/0

Vol. 172, No. 5

The 42- and 51-Kilodalton Mosquitocidal Proteins of *Bacillus sphaericus* 2362: Construction of Recombinants with Enhanced Expression and In Vivo Studies of Processing and Toxicity

ANDREW H. BROADWELL,† LINDA BAUMANN, AND PAUL BAUMANN*
Department of Microbiology, University of California, Davis, California 95616

Received 23 October 1989/Accepted 18 January 1990

After site-directed mutagenesis, the genes coding for the 42- and 51-kilodalton (kDa) mosquitocidal proteins of *Bacillus sphaericus* 2362 were placed under the regulation of the *aprE* (subtilisin) promoter of the *Bacillus subtilis* vector pUE (a derivative of pUB18). The levels of expression of the gene products in *B. subtilis* DB104 and *B. sphaericus* 718 were assessed by bioassays with larvae of *Culex pipiens* and by Western immunoblots. The results indicated that a higher amount of protein was produced in *B. subtilis* DB104. Electron microscopic examination of *B. subtilis* DB104 and *B. sphaericus* 718 containing the 42- and 51-kDa proteins indicated that amorphous inclusions accumulated in the former species and that crystals identical in appearance to that found in *B. sphaericus* 2362 were produced in the latter. Strains producing only the 42- or the 51-kDa protein were not toxic to larvae of *C. pipiens*. A mixture of both strains, a single strain producing both proteins, or a fusion of the 51- and the 42-kDa proteins was toxic. The amount of *B. subtilis* DB104 containing the 42- and the 51-kDa proteins necessary to kill 50% of the larvae of *C. pipiens* was 5.6 ng (dry weight) of cells per ml. This value was significantly lower than that for *B. sphaericus* 2362 (14 ng [dry weight] per ml). Larvae consuming purified amorphous inclusions containing the 42-kDa protein degraded this protein to primarily 39- and 24-kDa peptides, whereas inclusions with the 51-kDa protein were primarily degraded to a protein of 44 kDa. Past studies involving purified proteins from *B. sphaericus* 2362 indicate an association of toxicity with the 39-kDa peptide. The results presented here suggest that the 44-kDa degradation product of the 51-kDa protein may also be required for toxicity.

During sporulation, *Bacillus sphaericus* 2362 makes a parasporal crystal that is toxic for a variety of mosquito larvae (30). The crystal is composed of proteins having molecular masses of 51 and 42 kilodaltons (kDa) (5, 10). Both proteins have been purified from the *B. sphaericus* crystal, and only the 42-kDa protein is toxic for larvae of *Culex pipiens* (7). Upon ingestion of the crystal by mosquito larvae, the 51-kDa protein is rapidly degraded by larval gut proteases, whereas the 42-kDa protein is slowly converted to a protein of about 39 kDa (9). This processing results in a 54-fold increase in the toxicity of the protein for tissue culture grown cells of *Culex quinquefasciatus*. The larval gut proteases from at least four species of mosquito larvae are able to perform this proteolytic activation (9). The molecular basis of the mode of action of the *B. sphaericus* toxin is not known. Studies with the electron microscope and with fluorescein-labeled toxin have indicated that, as in the case of the lepidoptera- and diptera-active varieties of *Bacillus thuringiensis* (18), the site of action is midgut epithelial cells (11, 14, 30).

A 3.5-kilobase fragment containing what appears to be a single transcriptional operon coding for the 51- and 42-kDa proteins has been cloned into *Escherichia coli*, and its sequence has been determined (3, 5, 6, 17, 29). A low but significant sequence similarity has been observed between the 51- and 42-kDa proteins (5). The level of expression in *E. coli* of these two proteins was low. Recombinants producing only one of these proteins were not toxic to the larvae of *C. pipiens* (6). Recombinants producing both proteins in one cell or a mixture of recombinants producing each protein were toxic (6).

In this study, we have (i) used site-directed mutagenesis to construct recombinants having the genes for the 51- and 42-kDa proteins (singly and in combination) and recombinants containing fusions of the 51- and 42-kDa proteins, (ii) placed these genes under the control of the *aprE* (subtilisin) promoter and compared their expression in *Bacillus subtilis* DB104 and *B. sphaericus* 718, and (iii) studied the in vivo processing of these proteins in mosquito larvae.

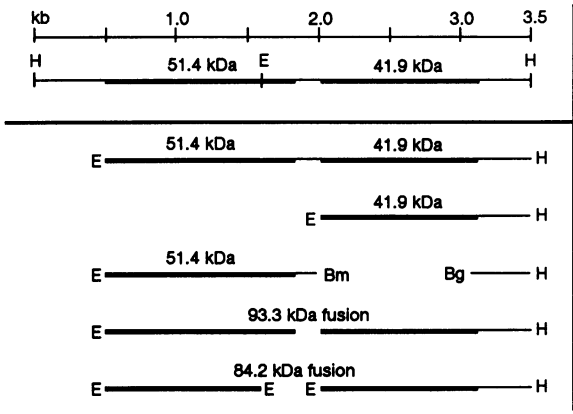
MATERIALS AND METHODS

Bacterial strains and plasmids. The origins of *B. sphaericus* 2362 and 718, *B. subtilis* DB104, *E. coli* JM105, and the *B. subtilis* vector pUB18 have been previously reported (2, 4, 6, 7, 10). *B. sphaericus* 718 is not toxic for mosquito larvae and does not make a parasporal crystal (22, 30).

General methods. The standard molecular biology methods used have been detailed by Maniatis et al. (24), and their application to our work has been described elsewhere (4, 6, 10). Additional methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblots have been described in our past publications (7, 8). All gels contained 12% (wt/vol) acrylamide. It should be noted that previous studies (5) found that the 51-kDa toxin protein migrates in gels at a position corresponding to about 63 kDa. In all figures, this protein band is designated as 51 kDa, although its position on the gel corresponds to 63 kDa. Unless otherwise noted, the antiserum used in the immunological studies was a mixture of antisera to the 42- and 51-kDa proteins of *B. sphaericus* (7).

In vitro mutagenesis. Our goal was to construct a series of DNA fragments bounded by unique *Eco*RI and *Hin*dIII (Fig.

* Corresponding author.
† Present address: DSIR, Plant Protection, Auckland, New Zealand.



pUE	Requires addition of 51.4 kDa	Requires addition of 41.9 kDa	*B. subtilis* DB104	*B. sphaericus* 718
382	—	—	5.6[a]	12
1-3a	+[b]	—	4.2	10
381	—	+[c]	22	48
384	—	—	7.2	92
389	+[b]	—	190	

FIG. 1. Constructs containing the genes coding for either or both of the 51- and 42-kDa proteins and their expression in different bacterial species, as determined by toxicity to larvae of *C. pipiens*. [a]LC_{50} values expressed as nanograms (dry weight) of bacterial cells per milliliter. [b]Bioassays performed in the presence of 0.5 μg (dry weight) of the same bacterial species containing the 51.4-kDa protein per ml. Cells with only the 42-kDa protein were not toxic at 420 μg (dry weight) of cells per ml (the highest concentration tested). [c]Bioassays performed in the presence of 0.5 μg (dry weight) of the same bacterial species containing the 41.9-kDa protein per ml. Cells with only the 51-kDa protein were not toxic at 420 μg (dry weight) of cells per ml (the highest concentration tested). Restriction sites: Bg, *Bgl*II; Bm, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. kb, Kilobases.

1) sites and containing either or both of the genes encoding the 51-kDa protein (gene 51) and the 42-kDa protein (gene 42). In addition, each of the genes was to be followed by a potential *B. sphaericus* terminator sequence (5). The *Eco*RI restriction site was to be positioned 3 base pairs (bp) upstream of the initiating ATG codon of either the 51- or the 42-kDa protein. The downstream end of the fragment was to contain the *Hin*dIII site. Such *Eco*RI-*Hin*dIII fragments could be cloned into expression vectors containing a promoter and a ribosome-binding site. We used the gapped duplex method for site-directed mutagenesis of Kramer et al. (20, 21) to obtain the desired constructs. The reagents were purchased as a kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the instructions provided by the manufacturer. A list of the oligonucleotides used, together with the relevant sequences, positions, and purposes of the site-directed mutageneses, is presented in Table 1.

Expression in *B. subtilis*. The *B. subtilis* vector pUB18, a derivative of pUB110 with an M13mp18 multiple cloning site, was modified by ligation of a 660-bp *Eco*RI-*Bam*HI fragment from pGR71SΔ4 (32) containing the sporulation-dependent subtilisin (*aprE*) σ^A (σ^{43}) promoter (27) and the beginning of the structural gene of this protease (32) (Fig. 2). To this fragment was ligated a 32-nucleotide-long adaptor (Fig. 2). This adaptor contained a *Bam*HI site at the upstream end and the single-stranded portion of the *Eco*RI site at the downstream end. Following the 17th amino acid from the subtilisin N terminus are three stop codons and a *B. subtilis* ribosome-binding site (15). Plasmids containing the *Eco*RI-*Hin*dIII inserts in this modified vector are designated by the prefix pUE followed by the construct designation.

The methods used for the cloning of genes in *B. subtilis* have been previously described (4, 32). For expression of the genes in *B. sphaericus* 718, the pUE plasmids containing the inserts were purified from *B. subtilis* and used to transform protoplasts as described by Hardy (16).

Preparation of cells. *B. subtilis* DB104 and *B. sphaericus* 718 containing pUE with inserts or pUB18 were grown at

TABLE 1. Oligonucleotides used for site-directed mutagenesis

Position[a]	Length (bp)	Relevant sequence[b]	Purpose
476	38	(489-GAATTCATG) AG	New *Eco*RI site 3 bp before 51.4-kDa protein
1584	32	(1597-GAGTTC) A	Removal of *Eco*RI site from sequence coding for 51.4-kDa protein
1989	34	(2001-GGATCC) G T	New *Bam*HI site in region between 51.4- and 41.9-kDa proteins
1996	31	2008-GAATTCATG A GA NonNon	New *Eco*RI site 3 bp before 41.9-kDa protein
3083	54	3103-TAATAAGATCT ATTATTAGAGA	Introduction of two stop codons and *Bgl*II site after 363rd amino acid of 41.9-kDa protein
1819	42	1819–1839, 2014–2034	Fusion of 51.4- and 41.9-kDa proteins

[a] Refers to position of the first nucleotide of the oligonucleotides relative to the sequence of the 3,479-bp *Hin*dIII DNA fragment from *B. sphaericus* 2362 containing the genes coding for the 51.4- and 41.9-kDa proteins (5).

[b] Top line gives the sequence of the proportion of the oligonucleotide of interest for this study; bottom line gives the original sequence of the *B. sphaericus* DNA. Parentheses indicate that the oligonucleotide used had the complementary sequence. Underlined sequence indicates a restriction site. ATG is the initiating methionine codon. Non, Nonsense codon.

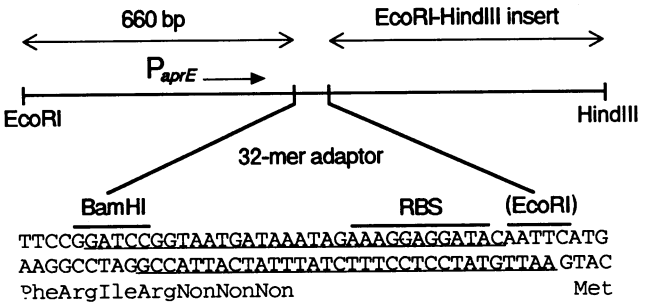


FIG. 2. Diagram of pUE (not drawn to scale) showing the arrangement of the fragment containing the *aprE* promoter and the beginning of the subtilisin structural gene, the 32-nucleotide adaptor, and the positions of the *Eco*RI-*Hin*dIII inserts. RBS, Ribosome-binding site; (*Eco*RI), ligation of the insert does not result in an *Eco*RI site; Non, nonsense codon. Arrow indicates direction of transcription.

32°C for 32 to 36 h in a complex medium suitable for sporulation (8).

Bioassays. The concentration (in dry weight of cells or in amount of crystal or inclusion body protein per milliliter) necessary to kill 50% of the larvae of *C. pipiens* (LC_{50}) was determined as described previously (7). The sole modification was the use of eggs that had been laid within a 12- to 14-h period and larvae that had hatched from these eggs at 44 to 48 h. Dilutions were made of the toxin preparation such that the relative amounts of 1, 0.75, 0.5, and 0.25 were maintained at each 10-fold dilution.

Purification of the *B. subtilis* inclusions. The methods used were those previously described for the purification of crystals from *B. sphaericus* 2362 (7). The sole modification was in the centrifugation of the French-pressed and washed preparation of inclusions and spores through a step gradient of 20, 30, 40, and 50% NaBr for 30 min. The material accumulating at the interphase of 40 and 50% NaBr was collected, washed, and used in bioassays. Protein was determined by the BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.), a modification of the method of Lowry et al. (23), with bovine serum albumin as the standard.

In vivo processing of the 42- and 51-kDa proteins. Purified inclusions from *B. subtilis* were fed to larvae, which after an appropriate time were harvested, ground, and centrifuged; the supernatant fraction and pellet were used for Western immunoblots. These methods have been described in detail elsewhere (7, 9).

Electron microscopy. *B. subtilis* DB104 and *B. sphaericus* 718 were grown as described above. Glutaraldehyde, to a final concentration of 0.3% (vol/vol), was added, and the suspension was placed on ice and shipped to M. F. Miller (Abbott Electron Microscopy Department) for processing and examination of thin sections with an electron microscope.

RESULTS

Synthesis of the 42- and 51-kDa proteins. Figure 1 shows a diagram of the constructs together with the LC_{50} values of the cells of *B. subtilis* DB104 and *B. sphaericus* 718 containing the 51- and 42-kDa proteins. Only cells of bacilli having recombinants with both gene 51 and gene 42 (pUE382) were toxic for larvae of *C. pipiens*. Cells having only the 42-kDa (pUE1-3a) or the 51-kDa (pUE381) protein were nontoxic; toxicity was observed when the assay mixture was supplemented with cells containing the complementary protein

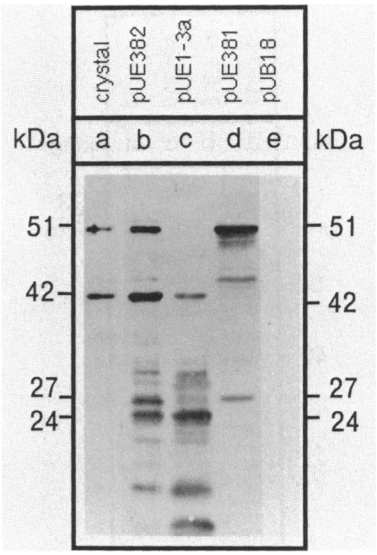


FIG. 3. Western immunoblot of extracts of *B. subtilis* DB104 containing the indicated recombinant plasmids. Bands were detected by antisera to the 42- and 51-kDa crystal proteins. For a diagram of the plasmids, see Fig. 1. Crystal, Purified crystals from *B. sphaericus* 2362. The amounts of samples (dry weight) used were as follows: lane a, 3 μg; lane b, 0.2 mg; lane c, 0.5 mg; lanes d and e, 0.7 mg.

(Fig. 1). In all cases, the LC_{50} values with *B. subtilis* DB104 were lower than those with *B. sphaericus* 718, suggesting a higher level of expression of the toxin proteins in the former species. Five different preparations of 32- to 36-h cultures of *B. subtilis* pUE382 were bioassayed; the LC_{50} for larvae of *C. pipiens* was found to be 5.6 ± 2.4 ng (dry weight) of cells per ml, a considerably better value than that obtained with *B. sphaericus* 2362 (14 ng/ml).

Western immunoblots of cell extracts from strains of *B. subtilis* DB104 containing the recombinant plasmids are presented in Fig. 3. The relative levels of the toxin proteins detected in the immunoblots were consistent with the amounts deduced from the bioassays. The principal bands of the recombinant-made proteins (lane b to d) migrated the same distance as the proteins from the crystal of *B. sphaericus* 2362 (lane a). Considerable degradation of the recombinant made protein was observed (lane b to d). The principal degradation product of the 42-kDa protein had a molecular mass of about 24 kDa (lane c). There appeared to be less degradation of the 51-kDa protein, with principal bands at 48 and 27 kDa (lane d). In the case of *B. sphaericus* 718, a similar pattern was observed in Western immunoblots (results not shown). A larger cell sample was, however, required to obtain toxin bands comparable to that obtained with *B. subtilis* DB104. This observation is consistent with the results of the bioassays (Fig. 1) and suggest that less protein was accumulated in *B. sphaericus* 718 than in *B. subtilis* DB104.

Synthesis of the fusion proteins. On the basis of the LC_{50} values (Fig. 1), the 93-kDa fusion protein (pUE384) was present in a higher amount in *B. subtilis* DB104 than in *B. sphaericus* 718. The LC_{50} of *B. subtilis* pUE384 (7.2 ng [dry weight] of cells per ml) was similar to that of *B. subtilis* pUE382 (5.6 ng/ml), which made both the 51- and 42-kDa proteins (Fig. 1). The 84-kDa fusion protein, consisting of a truncated 51-kDa protein fused to the 42-kDa protein, was not toxic alone and required the addition of cells containing

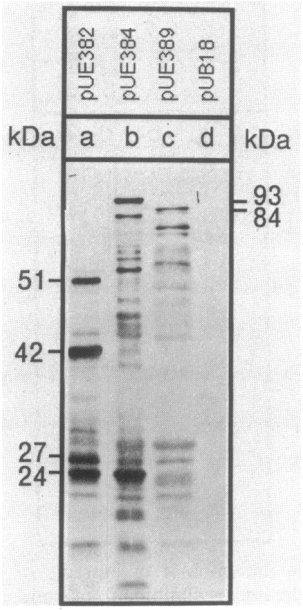


FIG. 4. Western immunoblot of extracts of *B. subtilis* DB104 containing the indicated recombinant plasmids. Bands were detected by antisera to the 42- and 51-kDa crystal proteins. See Fig. 1 for a diagram of the plasmids. The amounts of samples (dry weight) used were as follows: lanes a and b, 0.3 mg; lanes c and d, 1.2 mg.

the 51-kDa protein for toxicity (Fig. 1). A comparison of Western immunoblots of extracts of *B. subtilis* DB104 containing fusion proteins (Fig. 4, lane b and c) with that of extracts containing 42- and 51-kDa proteins (lane a) produced by pUE382 indicated the presence of degradation products having similar molecular masses.

Electron microscopy. The 51- and 42-kDa proteins produced by *B. subtilis* containing pUE382 accumulated within the cells as amorphous inclusions (Fig. 5a and b) that differed greatly from the ordered polyhedral structures (designated as crystals) characteristic of *B. sphaericus* (19, 31). In contrast, *B. sphaericus* 718 containing pUE382 produced inclusions with a structural periodicity (Fig. 5c) and appeared identical to the crystals of *B. sphaericus* (19, 31). In *B. subtilis* containing pUE1-3a (42-kDa protein), pUE381 (51-kDa protein), and pUE384 (93-kDa fusion protein), amorphous inclusions accumulated within the cells (results not shown). The inclusions formed in cells of *B. sphaericus* containing these recombinants were not examined by electron microscopy.

Properties of the purified amorphous inclusions of *B. subtilis*. Figure 6 presents the results of an electrophoretic analysis of the toxin proteins within the purified amorphous inclusions. Depending on the inclusion, there were different amounts of contaminating material. The cleanest preparations were those containing the 51- and 42-kDa proteins (Fig. 6, lane a, pUE382) and those with the 93-kDa fusion protein (lane d, pUE384). The most highly contaminated preparation was the inclusion body with the 51-kDa protein (lane c, pUE381). Western immunoblots of these preparations (lanes e to h) showed that unlike the whole cell extracts (Fig. 3, lanes b to d; Fig. 4, lane b), the inclusions were relatively free of degradation products of the toxin proteins.

The LC_{50} values obtained with the inclusion bodies containing the 51- and 42-kDa proteins (pUE382) and the 93-kDa fusion protein (pUE384) were 15 and 18 ng of BCA protein per ml, respectively, compared with 5.1 ng of BCA protein per ml for the purified crystal from *B. sphaericus* 2362.

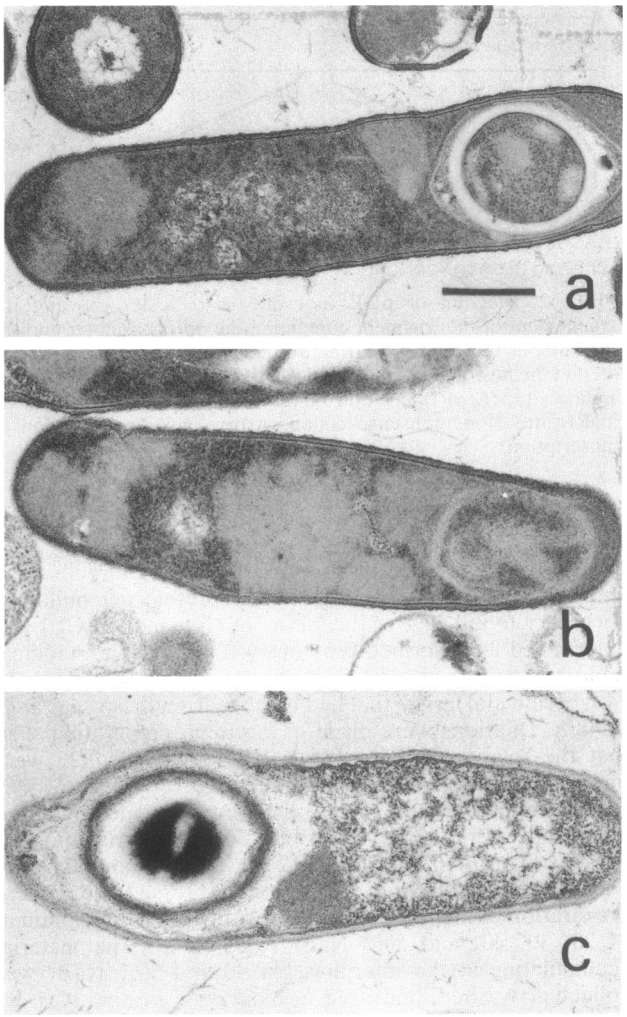


FIG. 5. Electron micrographs of *B. subtilis* DB104 (a and b) and *B. sphaericus* 718 (c) containing plasmid pUE382 with an insert coding for the 51- and 42-kDa proteins. Bar indicates 0.5 μm.

Attempts to purify crystals from *B. sphaericus* 718 were unsuccessful, since rupture of the cells by means of the French press also disrupted the spores into fragments that were not separable from the crystals on NaBr gradients.

Products of in vivo digestion of the inclusions. The amorphous inclusions (Fig. 6) were incubated with mosquito larvae, and after various times of feeding, the soluble inclusion-derived proteins found in the larvae were analyzed by means of Western immunoblots (Fig. 7). A variety of proteins of different molecular masses were detected by antisera to the 51- and 42-kDa proteins when inclusions containing both proteins and the 93-kDa fusion protein were fed to mosquito larvae (Fig. 7, lanes b and d). The principal bands of interest had molecular masses of 44, 41, 39, and 24 kDa; no bands corresponding to the undegraded proteins (42, 51, and 93 kDa) were detected. All of the degradation products except for the 44-kDa band were derived from the 42-kDa protein (lanes b and f). The principal degradation product of the 51-kDa protein appeared to be a protein of 44 kDa (arrow in lane c). This was confirmed by reacting immunoblots with antisera specific to either the 51-kDa protein (lanes g to j) or

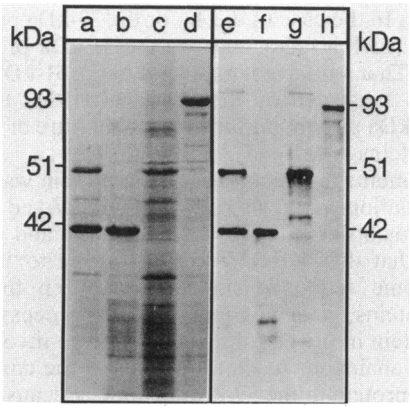


FIG. 6. Comparison of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (lanes a to d) and Western immunoblots (lanes e to h) of the amorphous inclusions isolated from *B. subtilis* DB104 containing recombinant plasmids. Lanes a to d were stained with Coomassie blue; lanes e to f were reacted with antibody to the 42- and 51-kDa crystal proteins. Protein was determined by the BCA reagent. Lanes: a, pUE382, 40 μg; b, pUE1-3a, 80 μg; c, pUE381, 160 μg; d, pUE384, 60 μg; e, pUE382, 0.8 μg; f, pUE1-3a, 1.6 μg; g, pUE381, 3.8 μg; h, pUE384, 1.4 μg.

the 42-kDa protein (lanes l to n). With antisera to the 51-kDa protein, all of the preparations tested (51 and 42 kDa [lane g], 51 kDa [lane h], and 93 kDa [lane i]) contained a principal degradation product of 44 kDa among other less prominent bands at higher and lower molecular masses. The amount of the 44-kDa protein was considerably decreased in samples taken after 1 h of feeding (results not shown). When these same preparations were reacted with antiserum specific for the 42-kDa protein, the 44-kDa degradation product was not detected, whereas the bands at 41, 39, and 24 kDa were present (lanes l to n).

DISCUSSION

The results presented above indicate that *B. subtilis* DB104 (Fig. 1, 3, and 4) and *B. sphaericus* 718 (Fig. 1)

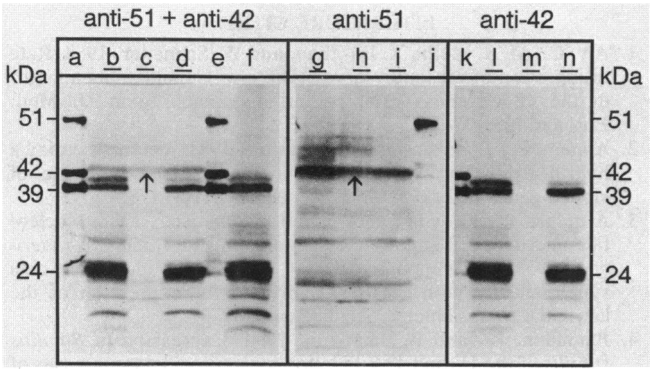


FIG. 7. Soluble proteins in the guts of larvae of *C. pipiens* detected by means of Western immunoblots after 30 min of feeding on the amorphous inclusions of Fig. 6. Lanes: a, e, j, and k, standards consisting of 2 μg of the purified 51- and 42-kDa crystal proteins of *B. sphaericus* 2362 and 1 μg of the purified 39-kDa protein derived from the 42-kDa protein of *B. sphaericus* 2362; b, g, and l, larvae fed inclusions containing 51- and 42 kDa proteins (pUE382); c, h, and m, larvae fed inclusions containing the 51-kDa protein (pUE381); d, i, and n, larvae fed inclusions containing the 93-kDa protein (pUE384); f, larvae fed inclusions containing the 42-kDa protein (pUE1-3a). Underlined letters indicate the same set of samples; arrow indicates the 44-kDa protein.

containing recombinant plasmids that produce the 51- and 42-kDa proteins or a 93-kDa fusion of these two proteins are toxic to larvae of *C. pipiens*. Strains that produce only the 42- or the 51-kDa protein are by themselves nontoxic. Toxicity is present when the two strains are combined. These observations confirm past results obtained with recombinants producing relatively low amounts of these proteins in *E. coli* (5, 6). Cells containing a fusion consisting of the N-terminal portion of the 51-kDa protein (with 79 amino acids deleted from the C terminus) and the 42-kDa protein were not toxic alone or when supplemented with cells containing the 42-kDa protein. Toxicity was restored by the 51-kDa protein, suggesting that all or a portion of the terminal 79 amino acids of the 51-kDa protein is required for toxicity.

From the results of the bioassays (Fig. 1) and the Western immunoblots, it is evident that more toxin proteins are produced in *B. subtilis* DB104 than in *B. sphaericus* 718. This observation is consistent with the fact that expression of the proteins in the pUE plasmids is regulated by the *B. subtilis* sporulation-dependent, *aprE* (subtilisin) promoter (27), which would be expected to function better in its own host than in *B. sphaericus*. In both species, considerable degradation of the recombinant-made proteins was found (Fig. 3 for *B. subtilis* DB104; not shown for *B. sphaericus* 718). It is not known whether the degradation products contributed to the toxicity of some preparations by remaining as aggregates and retaining toxicity, as may to be the case with the lepidoptera-active toxin of *B. thuringiensis* (28).

Electron microscopic examination of thin sections of *B. subtilis* DB104 containing the recombinant plasmids indicated massive accumulation of amorphous material lacking an obvious geometric shape or the structural periodicity characteristic of crystals of larvicidal strains of *B. sphaericus* (11, 30, 31). Amorphous inclusions of this sort are often found in cells overproducing a single protein (25). Such inclusions may consist of nonfunctional protein that must be renatured before function is restored. A comparison of the LC_{50} values obtained with a purified crystal preparation from *B. sphaericus* 2362 and the purified amorphous inclusions that contain both the 51- and 42-kDa proteins indicated that the former was approximately three times as toxic as the latter. This difference may indicate that a part of the protein in the amorphous inclusion is inactive. Alternatively, it should be noted that mosquito larvae are primarily particle eaters and exhibit preferences for certain particle sizes (12). Thus, comparisons involving different crystals and amorphous inclusions may give different values as a result of differences in the kinetics of uptake of the preparation (1). This may also be the explanation for the observation that the LC_{50} of the purified amorphous inclusions is higher than the LC_{50} of the whole cells that contain them.

Oeda et al. (26) reported that when *E. coli* contained a plasmid with the gene for the δ endotoxin of *B. thuringiensis* var. *aizawai*, temperature determined the appearance of the inclusions. When the cells were grown at 30°C, ordered bipyramidal crystals were produced; at 37°C, amorphous inclusions were formed. We have not examined the effect of temperature on the structure of the inclusions produced by the strains of bacilli used in our studies, all of which were grown at 32°C. Our results do, however, show that in the case of the 51- and 42-kDa proteins from *B. sphaericus*, the nature of the host determines the type of inclusion. In *B. subtilis* amorphous inclusions are formed, whereas in *B. sphaericus* 718 ordered crystals are produced (Fig. 6).

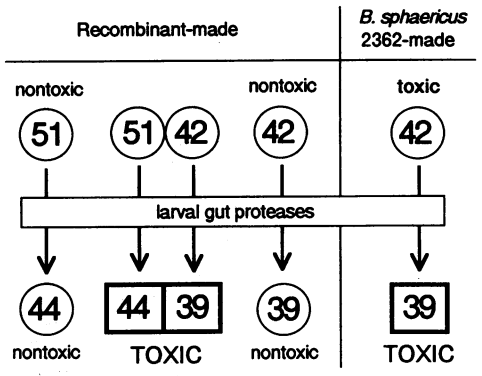


FIG. 8. Summary of the results of the in vivo processing of the recombinant-made 51- and 42-kDa proteins and the *B. sphaericus* 2362 crystal-derived 42-kDa protein by larvae of *C. pipiens*. In the latter case, conversion to the 39-kDa protein results in an increase in toxicity (9). Although the 51-kDa protein is required for toxicity of the recombinant-made 42-kDa protein, a direct role of the 44-kDa derivative is speculative. Numbers refer to molecular masses (in kilodaltons) of the proteins.

In our past studies, we have found that the 42-kDa *B. sphaericus* 2362 crystal-derived protein is toxic to mosquito larvae (7). After ingestion, this protein is converted by larval gut proteases to a 39-kDa peptide (9). This conversion results in a 54-fold increase in the toxicity of the protein for tissue culture-grown cells of *C. quinquefasciatus*. No toxicity was associated with the 51-kDa protein (7). These results differ from those obtained with the recombinant-made 42-kDa protein, which is not toxic unless accompanied by the 51-kDa protein (this study; 5, 6). A diagram summarizing these results is presented in Fig. 8. Previously, a number of suggestions were made to account for this difference in the behavior of the crystal-derived and recombinant-made 42-kDa protein (5). It was noted that the 42-kDa protein isolated from the crystal lacked the first four amino acids, and it was suggested that the removal of these four amino acids by a *B. sphaericus* protease was essential for making the 42-kDa protein susceptible to the action of larval gut proteases which activate it to the 39-kDa peptide (5). It was also suggested that the 51-kDa protein may provide an accessory function for the 42-kDa recombinant-made protein which substitutes for its activation to the 39-kDa peptide, thereby accounting for the requirement of both recombinant-made proteins for toxicity (5).

The results of this study provide new information that eliminates some of these suggestions. The finding that *B. sphaericus* 718 containing only the 42-kDa protein is alone not toxic suggests that the host does not contribute a processing step essential for toxicity. The results of the in vivo experiments with mosquito larvae (summarized in Fig. 8) show that the 42-kDa recombinant-made protein is readily converted to the 39-kDa protein (Fig. 7); therefore, the lack of toxicity cannot be explained by the inability of the larvae to generate this peptide. This result also indicates that processing of the 42-kDa protein to the 39-kDa protein is by itself insufficient to activate the toxin. The existence of additional factors besides proteolysis that are essential for conversion of a toxin to an active form has been inferred in the case of botulinum toxin (13). This protein is made as a single peptide that is nicked to give the dimeric structure characteristic of the mature toxin. Whether the resulting structure is fully activated depends on as yet unknown factors that appear to cause a conformational change in the protein (13). In the case of the 42- or the 39-kDa protein of *B. sphaericus*, such a factor may be the 51-kDa protein or its derivative. That a transient exposure to the 51-kDa protein is sufficient is suggested by the observation that the crystal-derived 42-kDa protein purified from a mixture of the 51- and 42-kDa proteins is toxic (7).

In experiments in which the 51-kDa protein was ingested, an accumulation of a 44-kDa protein derived from this molecule was observed (Fig. 7, lanes b to d and g to i), and it may be that this derivative has a role in toxicity. Since there is some sequence similarity between the 51- and 42-kDa proteins, it is possible that the processing of the 51-kDa protein in the gut of mosquito larvae involves cleavage at sites analogous to those cleaved in the conversion of the 42-kDa protein to the 39-kDa peptide. Because the mode of action of the *B. sphaericus* toxin at the molecular level is not known, one can only speculate as to the possible role of the 51-kDa protein or its derivative, the 44-kDa peptide. The results with the *B. sphaericus* 2362 crystal-derived protease-treated 42-kDa protein (purified from a mixture of the 51- and 42-kDa proteins) indicate that the resulting 39-kDa protein is alone sufficient for toxicity (9). A possible function for the 51-kDa protein or its derivative the 44-kDa protein is suggested by the recent discoveries of a number of "molecular chaperones" whose function appears to be the direction of proper folding of proteins (25). It is possible that their function is a transient one allowing the folding of the 42- or the 39-kDa protein into a conformation essential for toxicity. These predictions should be tested with purified proteins and mosquito-derived tissue culture-grown cells. The amorphous inclusions containing the 42- or 51-kDa protein or both provide the starting material for such studies.

ACKNOWLEDGMENTS

We are grateful to David R. Wilcox for advice and assistance and to Buzz Miller and Figen Durum for the electron micrographs.

This research was supported by Abbott Laboratories, the Vector Biology Components of United Nations Development Program/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases, and the University of California Mosquito Research Program.

LITERATURE CITED

1. **Aly, C., M. S. Mulla, X. Bo-Zhao, and W. Schnetter.** 1988. Rate of ingestion of mosquito larvae (Diptera: Culicidae) as a factor in the effectiveness of a bacterial stomach toxin. J. Med. Entomol. **25**:191–196.
2. **Amann, E., J. Brosius, and M. Ptashne.** 1983. Vectors bearing a hybrid trp-lac promoter useful for regulating expression of cloned genes in *Escherichia coli*. Gene **25**:167–178.
3. **Arapinis, C., F. de la Torre, and J. Szulmajster.** 1988. Nucleotide and deduced amino acid sequence of the *Bacillus sphaericus* 1593M gene encoding a 51.4 kD polypeptide which acts synergistically with the 42 kD protein for expression of the larvicidal toxin. Nucleic Acids Res. **16**:7731.
4. **Baumann, L., and P. Baumann.** 1989. Expression in *Bacillus subtilis* of the 51- and 42-kilodalton mosquitocidal toxin genes of *Bacillus sphaericus*. Appl. Environ. Microbiol. **55**:252–253.
5. **Baumann, L., A. H. Broadwell, and P. Baumann.** 1988. Sequence analysis of the mosquitocidal toxin genes encoding 51.4- and 41.9-kilodalton proteins from *Bacillus sphaericus* 2362 and 2297. J. Bacteriol. **170**:2045–2050.
6. **Baumann, P., L. Baumann, R. D. Bowditch, and A. H. Broadwell.** 1987. Cloning of the gene for the larvicidal toxin of *Bacillus sphaericus* 2362: evidence for a family of related sequences. J. Bacteriol. **169**:4061–4067.
7. **Baumann, P., B. M. Unterman, L. Baumann, A. H. Broadwell, S. J. Abbene, and R. D. Bowditch.** 1985. Purification of the larvicidal toxin of *Bacillus sphaericus* and evidence for high-

molecular-weight precursors. J. Bacteriol. **163**:738–747.
8. **Broadwell, A. H., and P. Baumann.** 1986. Sporulation-associated activation of *Bacillus sphaericus* larvicide. Appl. Environ. Microbiol. **52**:758–764.
9. **Broadwell, A. H., and P. Baumann.** 1987. Proteolysis in the gut of mosquito larvae results in further activation of the *Bacillus sphaericus* toxin. Appl. Environ. Microbiol. **53**:1333–1337.
10. **Bowditch, R. D., P. Baumann, and A. A. Yousten.** 1988. Cloning and sequencing of the gene encoding a 125-kilodalton surface layer protein from *Bacillus sphaericus* 2362 and a related cryptic gene. J. Bacteriol. **171**:4178–4188.
11. **Charles, J.-F.** 1987. Ultrastructural midgut events in Culicidae larvae fed with *Bacillus sphaericus* 2297 spore/crystal complex. Ann. Inst. Pasteur (Paris) **138**:471–484.
12. **Dadd, R. H.** 1975. Ingestion of colloid solutions by filter-feeding mosquito larvae: relationship to viscosity. J. Exp. Zool. **191**: 395–406.
13. **DasGupta, B. R.** 1989. The structure of butulinum neurotoxin, p. 53–67. *In* L. L. Simpson (ed.), Botulinum neurotoxin and tetanus toxin. Academic Press, Inc. San Diego, Calif.
14. **Davidson, E. W.** 1988. Binding of the *Bacillus sphaericus* (Eubacteriales: Bacillaceae) toxin to midgut cells of mosquito (Diptera: Culicidae) larvae: relationship to host range. J. Med. Entomol. **25**:151–157.
15. **Hager, P. W., and J. C. Rabinowitz.** 1985. Translational specificity in *Bacillus subtilis*, p. 1–32. *In* D. A. Dubnau (ed.), The molecular biology of the bacilli, vol. 2. Academic Press, Inc., New York.
16. **Hardy, K. G.** 1985. *Bacillus* cloning methods, p. 1–17. *In* D. M. Glover (ed.), DNA cloning, vol. 2. IRL Press, Oxford.
17. **Hindley, J., and C. Berry.** 1987. Identification, cloning and sequence analysis of the *Bacillus sphaericus* 1593 41.9 kD larvicidal toxin gene. Mol. Microbiol. **1**:187–194.
18. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53**:242–255.
19. **Kalfon, A., J.-F. Charles, C. Bourgouin, and H. de Barjac.** 1984. Sporulation of *Bacillus sphaericus* 2297: an electron microscope study of crystal-like inclusion biogenesis and toxicity to mosquito larvae. J. Gen. Microbiol. **130**:893–900.
20. **Kramer, W., V. Drutsa, H.-W. Jansen, B. Kramer, M. Pflugfelder, H.-J. Fritz.** 1984. The gapped duplex DNA approach to oligonucleotide-directed mutagenesis. Nucleic Acids Res. **12**:9441–9456.
21. **Kramer, W., and H.-J. Fritz.** 1987. Oligonucleotide-directed construction of mutations via gapped duplex DNA. Methods Enzymol. **154**:350–367.
22. **Krych, V. K., J. L. Johnson, and A. A. Yousten.** 1980. Deoxyribonucleic acid homologies among strains of *Bacillus sphaericus*. Int. J. Syst. Bacteriol. **30**:476–484.
23. **Lowry, O. H., J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mitraki, A. M., and J. King.** 1989. Protein folding intermediates and inclusion body formation. Bio/Technology **7**:690–697.
26. **Oeda, K., K. Inouye, Y. Ibuchi, K. Oshie, M. Shimizu, K. Nakamura, R. Nishioka, Y. Takada, and H. Ohkawa.** 1989. Formation of crystals of the insecticidal proteins of *Bacillus thuringiensis* subsp. *aizawai* IPL7 in *Escherichia coli*. J. Bacteriol. **171**:3568–3571.
27. **Park, S.-S., S.-L. Wong, L.-F. Wang, and R. H. Doi.** 1989. *Bacillus subtilis* subtilisin gene (*aprE*) is expressed from a σ^A (σ^{43}) promoter in vitro and in vivo. J. Bacteriol. **171**:2657–2665.
28. **Schnepf, H. R., and H. R. Whiteley.** 1985. Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. J. Biol. Chem. **260**:6273–6280.
29. **Tandeau de Marsac, N., F. de la Torre, and J. Szulmajster.** 1987. Expression of the larvicidal gene of *Bacillus sphaericus* 1593M in the cyanobacterium *Anacyctis nidulans* R2. Mol. Gen. Genet. **209**:396–398.
30. **Yousten, A. A.** 1984. *Bacillus sphaericus*: microbiological factors related to its potential as a mosquito larvicide. Adv. Biotechnol. Processes **3**:315–343.
31. **Yousten, A. A., and E. W. Davidson.** 1982. Ultrastructural analysis of spores and parasporal crystals formed by *Bacillus sphaericus* 2297. Appl. Environ. Microbiol. **44**:1449–1455.
32. **Zaghloul, T., F. Kawamura, and R. H. Doi.** 1985. Translational coupling in *Bacillus subtilis* of a heterologous *Bacillus subtilis-Escherichia coli* gene fusion. J. Bacteriol. **164**:550–555.