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Bacillus sphaericus as a Mosquito Pathogen: Properties of the Organism and Its Toxins†

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INTRODUCTION

In the course of sporulation, some strains of *Bacillus sphaericus* synthesize a parasporal inclusion or "crystal" (Fig. 1), which contains proteins toxic for larvae of a variety of mosquito species (64, 74). Upon completion of sporulation, the crystal remains associated with the endospore, both being enclosed within the exosporium (74). The major components of the crystal are two proteins of 51 and 42 kDa (12); neither protein alone is toxic to larvae, and both are required for toxicity (11, 20, 23). Following ingestion of the crystal by mosquito larvae, these two proteins are solubilized in the midgut (19, 74). Electron-microscopic studies have indicated the midgut epithelial cells as the primary target of the toxin (24, 74). *B. sphaericus* has been successfully used for the biological control of mosquito species which are vectors of important human diseases (56, 74).

A number of other bacilli resemble *B. sphaericus* in producing parasporal crystals (6). The most extensively studied species is *Bacillus thuringiensis* (6, 52, 72). Members of this species produce a variety of plasmid-encoded proteins which accumulate during sporulation to form a crystal. Upon completion of sporulation, the crystal and the endospore usually separate. In general, crystals of *B. thuringiensis* contain proteins of 125 to 140 kDa or 70 to 80 kDa (52), and, depending on the variety of this species, the proteins may be toxic to insects of the orders Lepidoptera (caterpillars), Coleoptera (beetles), or Diptera (mosquitos and blackflies). Upon ingestion by a susceptible species, the proteins in these crystals are solubilized in the midgut by a combination of alkaline pH and proteolysis (6, 70). The high-molecular-weight proteins are protoxins; proteolysis in the gut leads to their conversion to lower-molecular-weight toxins (6, 72). Although synergism among the various *B. thuringiensis* toxin proteins may be important, individual proteins are toxic when tested in a susceptible host (27, 52).

In addition to *B. sphaericus*, *B. thuringiensis* subsp. *israelensis* is used for the biological control of mosquitos (56). This variety differs from *B. sphaericus* in the nature of its toxins and its host range (12, 52, 74). In general, *B. sphaericus* is more active against *Culex* and *Anopheles* spp. and less active against *Aedes* spp., whereas *B. thuringiensis* subsp. *israelensis* is more active against *Aedes* and *Culex*

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† Dedicated to the memory of Helen R. Whiteley.

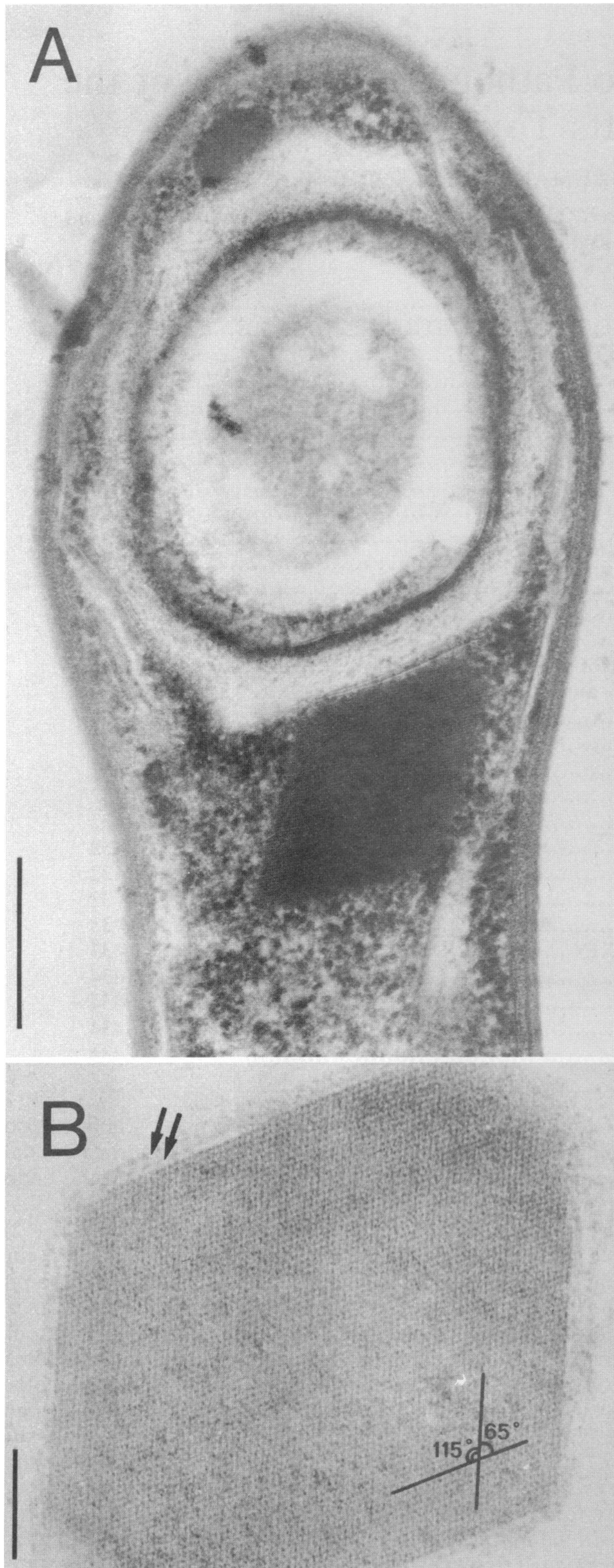

FIG. 1. Electron micrographs of *B. sphaericus* 2297. (A) Sporulating cell containing crystal. Bar, 400 nm. Reprinted with permission from reference 75. (B) Higher magnification of crystal showing internal structure. Arrows indicate two layers of the envelope which surround the crystal. Bar, 200 nm. Reprinted with permission from reference 42.

spp. and less active against *Anopheles* spp. (56). *B. thuringiensis* subsp. *israelensis* is also active against blackflies, whereas *B. sphaericus* is not (56). *B. sphaericus* has an additional, useful attribute in its ability to persist in polluted aquatic environments in which the toxicity of *B. thuringiensis* subsp. *israelensis* is rapidly lost (41).

In the present review we will summarize the recent literature on *B. sphaericus* with major emphasis on the larvicidal toxins of this species. Where appropriate, these toxins will be compared with those of *B. thuringiensis*. An extensive review of the literature prior to 1984 has been provided by Yousten (74). A more recent review dealing with practical aspects of *B. sphaericus* is that of Singer (68).

TAXONOMY AND GENERAL PHYSIOLOGY OF MOSQUITOCIDAL STRAINS

The principal phenotypic traits used to place strains into the species *B. sphaericus* are the presence of spherical spores, the inability to grow anaerobically, and a negative reaction on a variety of tests developed primarily for the classification of the family *Enterobacteriaceae* (30, 74). In view of the fact that *B. sphaericus* is defined by the absence of positive traits, it is not surprising that in vitro DNA-DNA hybridization studies indicated that the species is highly heterogeneous and contains DNA homology groups deserving distinct species status (55). At the time of the in vitro DNA-DNA hybridization study (1980), these groups were not phenotypically distinguishable, so they were not given separate species designations. Recently, broader taxonomic approaches involving an expanded nutritional screening and a numerical analysis of the data indicated that these strains constitute a number of distinct clusters, in agreement with the results of DNA homology studies (2). Since the clusters are not readily distinguishable by universally positive and negative traits, further studies with more strains and additional traits are necessary before these clusters can be given species designations.

Mosquitocidal strains of *B. sphaericus* can be divided into two groups on the basis of their toxicity to mosquito larvae (Table 1). Strains which are highly toxic make a parasporal crystal, whereas strains with low toxicity lack a crystal. The high- and the low-toxicity strains are related by DNA homology values of over 79%, a finding consistent with their placement into a single species (55). These strains have been placed into DNA homology group IIA, which is 57 to 66% related to DNA homology group IIB, consisting of nontoxic strains. These two closely related homology groups have also been differentiated by a numerical analysis of their phenotypic properties, although only a few diagnostic traits are useful for their identification (2). Strain 718 of DNA homology group IIB has been used as a host for the expression of crystal proteins from *B. sphaericus* 2362 (19, 20).

Strain ATCC 14577, the type strain of *B. sphaericus*, is related to the mosquitocidal strains by DNA homology values of 14 to 25% (55) and is also phenotypically distinct from these isolates (2). These results indicate that the epithet *sphaericus* does not belong to the mosquito pathogens, which deserve a new species designation.

Primarily with the intent of identifying strains in ecological studies, a system of flagellar serotyping and bacteriophage typing has been developed for the mosquitocidal strains of *B. sphaericus* (43, 45, 73, 76). Some of the antisera, as well as some of the bacteriophages, react with nontoxic strains from other DNA homology groups (76).

TABLE 1. Properties of the more extensively studied mosquitocidal strains of *B. sphaericus*[a]

Strain designation	Crystal	Toxicity[b]	DNA homology group[c]	Flagellar serotype	Bacterio-phage group	51- and 42-kDa proteins detected[d]	Gene-51 and gene-42 detected by hybridization[e]	Gene-51 and gene-42 sequenced
Kellen K	–	L	IIA	1	1	–	–	ND[f]
SSII-1	–	L	IIA	2a2b	2	–	–	ND
1593	+	H	IIA	5a5b	3	+	+	+
1691	+	H	IIA	5a5b	3	+	+	ND
2362	+	H		5a5b	3	+	+	+
2297	+	H		25	4	+	ND	+
2317.3	+	H		5a5b		ND	ND	+
IAB59	+	H		6	3	+	ND	+
718	–	–	IIB	6		–	–	ND

[a] Data compiled from references 10, 11, 14, 43, 44, 45, 55, 73, and 76. –, Present; +, absent.
[b] Effect on larvae of *C. pipiens*. L, low toxicity (LC_{50} > 5 μg [dry weight] of cells per ml); H, high toxicity (LC_{50} < 30 ng [dry weight] of cells per ml).
[c] Strains in group IIA are related by over 79% DNA homology; groups IIA and IIB are related by 57 to 66% DNA homology (55).
[d] Detected by antisera in Western immunoblots or by Ouchterlony immunodiffusion.
[e] Detected in Southern blots.
[f] ND, not determined.

The larvicidal strains of *B. sphaericus* share a number of properties (2, 10). All are strict aerobes and are unable to ferment glucose, denitrify, or reduce nitrate to nitrite. Extracellular enzymes such as amylase, gelatinase, chitinase, and lecithinase are lacking. None are able to utilize pentoses, hexoses, or disaccharides as sources of carbon and energy; all are able to utilize gluconate. Most strains utilize a variety of carbon compounds, which include fatty acids, tricarboxylic acid intermediates, and amino acids. A majority of the strains are able to utilize adenine (2). All strains grow at 40°C and have growth factor requirements which may be satisfied by biotin and thiamine (74). The G+C content of the DNA is 35 to 37 mol% (30, 55).

The inability to utilize glucose is due to the absence of an uptake system as well as the early enzymes of the Embden-Meyerhof and Entner-Doudoroff pathways (65). Gluconate appears to be utilized via the pentose phosphate shunt.

GENERAL PHYSIOLOGY AND BIOCHEMISTRY OF THE CRYSTAL

Synthesis

The crystal of *B. sphaericus* is a parallelepiped (42). The interior shows a crystalline lattice structure with striations about 6.3 nm apart (Fig. 1B) (42, 75). The crystal is surrounded by an envelope (Fig. 1B) (42, 75) similar in appearance to that surrounding the crystals of *B. thuringiensis* (49). The envelope appears to be retained upon dissolution of the crystal matrix in the larval gut or after treatment with alkali (42, 75).

The relation between growth, sporulation, crystal formation, and toxicity for mosquito larvae has been studied for strains 1593 (59), 2297 (54, 75), and 2362 (21). Crystal formation was detected immediately following septum formation by the sporulating cell, corresponding to the beginning of stage III in the sporulation cycle (54, 75). This observation suggests that the crystal of *B. sphaericus* is formed slightly earlier than the crystal of *B. thuringiensis* subsp. *kurstaki* (13). Toxicity to larvae paralleled the appearance and growth of the crystal. The 51- and 42-kDa proteins were absent in the exponential phase of growth and appeared in approximately equal amounts during sporulation (21, 26). The association between crystal formation and sporulation was further established by studies of oligosporogenic mutants (26, 59). Mutants blocked at early stages of sporulation failed to make crystal or crystal proteins and were nontoxic, whereas mutants blocked at late stages of sporulation contained crystals and crystal proteins and were toxic (26).

Purification and Properties of Crystal Proteins

Payne and Davidson (64) disrupted the exosporium-enclosed crystal-spore complex of *B. sphaericus* 1593 by passage through a French pressure cell and separated the crystals from the spores by centrifugation through NaBr gradients. Toxicity for larvae was associated with a fraction containing the crystal. The most toxic preparation was, however, not pure, being contaminated with membranous fragments presumably derived from the sporangium or the exosporium (64). With slight modifications, this approach was used for the subsequent purification of crystals or inclusion bodies from different strains.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of partially purified crystal preparations from strain 2362 indicated that the major constituents were proteins of 122, 110, 51, and 42 kDa (12). The 122- and 110-kDa proteins were subsequently shown to be contaminants originating from the surface layer protein of *B. sphaericus* (17). These proteins will not be considered here, since it has recently been shown that their toxicity to mosquito larvae is due to contamination with small amounts of the 51- and 42-kDa proteins (8). The crystal preparation was solubilized by treatment with alkali (33), and the 51- and 42-kDa proteins were purified to electrophoretic homogeneity by gel filtration through Sephadex G-200 followed by DEAE-agarose chromatography and gel filtration through Bio-Rad P-100 or P-60 (12). The N-terminal sequence of 40 amino acids of the 42-kDa protein was determined; the 51-kDa protein had a variety of N termini, thereby precluding sequence determination. Antisera prepared to the 51- and 42-kDa proteins were used in Ouchterlony immunodiffusion experiments and Western immunoblots. The lack of immunological cross-reaction between the 51- and the 42-kDa proteins indicated that they are antigenically distinct. Proteins immunologically related to the 51- and the 42-kDa proteins of *B. sphaericus* 2362 were detected in other highly toxigenic strains of this species, but not in strains which had low or no larvicidal activity (Table 1). The 42-kDa protein has also been purified from strain 1593 (60, 67).

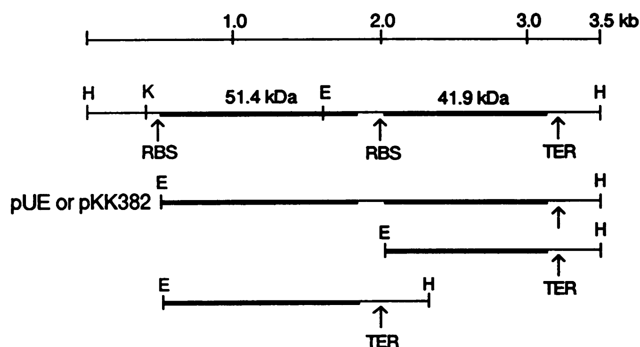

FIG. 2. Map of the 3.5-kb *Hin*dIII fragment containing the genes for the 51- and 42-kDa proteins. Abbreviations: TER, terminator; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I.

A major problem in the purification of the 51- and the 42-kDa proteins was their tendency to form aggregates (12). This may be explained by their difference in charge; at pH 7.8, the 51-kDa protein migrated to the cathode, while the 42-kDa protein migrated to the anode (12).

GENETICS OF CRYSTAL PROTEINS

Cloning and Nucleotide Sequence Analysis

Using synthetic oligonucleotide probes designed on the basis of the N-terminal sequence of the 42-kDa protein (12) and a genomic library of *B. sphaericus* 1593, Hindley and Berry (51) cloned the gene for the 42-kDa protein into *Escherichia coli* and determined its sequence. Baumann et al. (9, 11) cloned into *E. coli* and sequenced a 3.5-kb *Hin*dIII fragment from strain 2362 which contained the genes for the 51- and the 42-kDa proteins. Subsequently, the sequence of similar DNA fragments was determined from four additional, highly toxigenic strains (Table 1) (14). The nucleotide sequence is highly conserved, being identical in strains 1593, 2317.3, and 2362. Strains 2297 and IAB59 differ by 25 and 7 nucleotides, respectively, from the preceding strains. Using a hybridization probe derived from part of the gene coding for the 51-kDa protein and all of the gene coding for the 42-kDa protein, Baumann et al. showed that strain 1691 contains both genes, whereas two strains of low larvicidal potency do not (Table 1) (11).

All of the 3.5-kb DNA fragments have the same genetic organization, which is diagramed in Fig. 2. The open reading frame (ORF) coding for the 51-kDa protein is preceded by a ribosome-binding site (RBS). Following the stop codon which terminates the 51-kDa protein is a spacer region of 174 to 176 nucleotides containing several additional stop codons and an RBS preceding the ORF coding for the 42-kDa protein. Downstream of the ORF coding for the 42-kDa protein is a sequence consisting of a G+C-rich hairpin loop followed by a row of Ts characteristic of transcription terminators. This organization suggests that the genes for the 51- and 42-kDa proteins are in a single transcription unit. The nucleotides upstream of the coding region for the 51-kDa protein did not contain a sequence readily identified as similar to one of the *Bacillus subtilis* sporulation promoters (58).

Louis et al. (57) and Ganesan et al. (50) have previously claimed to have cloned and expressed the *B. sphaericus* mosquitocidal toxin gene in *E. coli*. Their restriction map does not match any of the sites of the 3.5-kb *Hin*dIII fragment. Subsequently, it was noted that the "larvicidal activity gradually decreased with time, although no apparent deletion was observed" (47). From the data presented it is clear that their initial clone is not related to the DNA fragment containing the *B. sphaericus* crystal protein genes.

Location of Crystal Genes

There is no definitive evidence for the location of crystal genes either on plasmids or on the bacterial chromosome. The most thorough study of the plasmid content of *B. sphaericus* isolates is that of Singer (68), who found a 75-MDa plasmid in many larvicidal and nonlarvicidal strains. In addition, some strains had plasmids of lower molecular weight. One highly toxic strain (strain 1691) (Table 1) lacked detectable plasmids, an observation suggesting that the toxin genes are chromosomal. The conclusion of Louis et al. (57) that the genes for the toxin are chromosomal is not valid, since the probe used was not to the crystal toxin genes.

Expression of Crystal Genes in Different Bacterial Hosts

The genes on the 3.5-kb fragment coding for the 51- and the 42-kDa proteins (Fig. 2) were expressed at low levels in *E. coli* during vegetative growth (11). Similarly, the genes on this fragment were expressed in the blue-green bacterium *Anacystis nidulans* (47). The *Kpn*I-*Hin*dIII fragment (Fig. 2), which includes 96 bp preceding the ORF corresponding to the 51-kDa protein, was ligated into pUB18 (a derivative of pUB110) and used to transform several *Bacillus* species. The 51- and 42-kDa proteins were not present during vegetative growth, but appeared in the course of sporulation in *B. subtilis* DB104 (7), *B. sphaericus* 718, and *B. sphaericus* SSII-1 (20). These results indicate that all or part of the sporulation-dependent *B. sphaericus* promoter which regulates the transcription of the 51- and 42-kDa protein genes is contained in the 96 bp upstream of the gene coding for the 51-kDa protein. Similarly, when the 3.5-kb *Hin*dIII fragment (Fig. 2) was cloned into *B. thuringiensis* subsp. *israelensis*, the 51- and 42-kDa proteins appeared during sporulation (16).

PROPERTIES OF CRYSTAL PROTEINS

Analysis of Deduced Amino Acid Sequence

The two major ORFs on the 3.5-kb *Hin*dIII fragment (Fig. 2) code for proteins of 448 and 370 amino acids with deduced molecular masses of 51 and 42 kDa, respectively. These genes from five strains of *B. sphaericus* have been sequenced (Table 1). Three contain identical 51- and 42-kDa proteins; two have 51-kDa proteins that differ by 3 to 5 amino acids and 42-kDa proteins that differ by 1 to 5 amino acids (14). Most of these changes involve substitutions of similar amino acids. The high sequence conservation of the *B. sphaericus* proteins is in contrast to the insecticidal proteins of *B. thuringiensis* (52). Depending on the variety of this species, considerable sequence divergence has been found. Despite these differences, it is clear that most of the *B. thuringiensis* insecticidal proteins belong to a single protein family (52). A comparison of the amino acid sequences of the 51- and 42-kDa proteins with the sequences of representative *B. thuringiensis* crystal proteins active against members of the orders Diptera, Lepidoptera, or Coleoptera showed no significant sequence similarity (9),

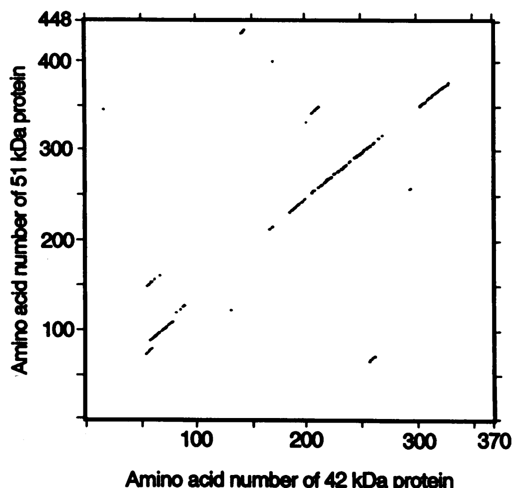

FIG. 3. Dot-matrix comparison showing the amino acid sequence similarity of the 51- and 42-kDa proteins. The window was 10 amino acids, and the stringency was 60%.

thereby indicating that the 51- and 42-kDa proteins of *B. sphaericus* constitute a separate family of insecticidal toxins.

The 51- and 42-kDa proteins have a low but significant sequence similarity (Fig. 3). Alignment of the two protein sequences from strain 2362 indicates amino acid identity at 93 positions or 25% sequence similarity within the 370 common positions. There are, however, four segments of the proteins where the amino acid sequence is highly conserved. Figure 4 presents a hydrophobicity profile of the two proteins and the positions of the four highly conserved regions. Two of these segments (segments 1 and 2) are hydrophobic and consequently may be involved in interactions with membranes. The sequence similarity between the 51- and the 42-kDa proteins suggests that they came from a single ancestor and arose from a gene duplication followed by sequence divergence and, possibly, functional specialization.

As indicated above, the 51- and the 42-kDa proteins differ in their charge, as shown by their opposite electrophoretic mobilities in agarose gels (12). A calculation of the approximate charge of these proteins at pH 7.0 indicates a charge

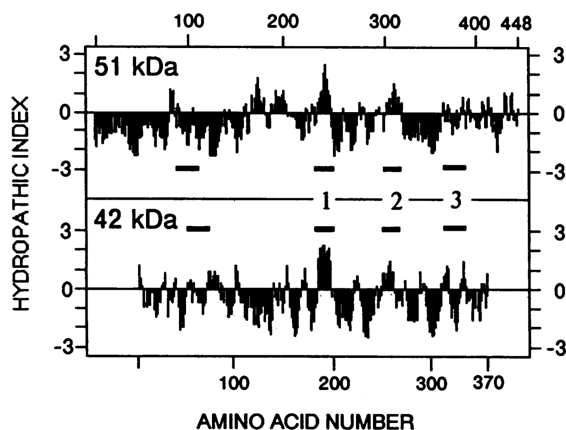

FIG. 4. Hydrophobicity profile of the 51- and 42-kDa proteins. Bars indicate the four major conserved regions. Numbers between bars designate regions which were deleted in subsequent studies.

difference of 8, with the 42-kDa protein being more negatively charged. This observation is consistent with the migration of the 42-kDa protein to the anode.

Overexpression of Crystal Proteins in Different Hosts

Construction of recombinants. Although in the initial cloning experiments the genes for the 51- and 42-kDa proteins were expressed in *E. coli* as indicated by immunoblots and toxicity to larvae, the levels were low and attempts at purification were unsuccessful (9, 11). To increase the amount of protein produced, two expression vectors were used. The first, pUE, is a derivative of the *B. subtilis* vector pUB18 (19) modified to contain the *aprE* (subtilisin) promoter (63) followed by a *B. subtilis* RBS and an *Eco*RI site. The *aprE* promoter is transcribed early in sporulation (63). The second is an *E. coli* expression vector, pKK223-3, regulated by the *tac* promoter, which also contains an RBS followed by an *Eco*RI restriction site (5). The *tac* promoter is induced by isopropyl-β-D-thiogalactoside during exponential growth of the culture. By using site-directed mutagenesis, *Eco*RI restriction sites were placed in front of the initiating codons of the 51- and 42-kDa proteins (Fig. 2). These modifications allowed the placement of an *Eco*RI-*Hin*dIII fragment immediately after the RBS of the appropriate vector. The constructs could synthesize both the 51- and the 42-kDa proteins, only the 51-kDa protein, or only the 42-kDa protein (Fig. 2) (19, 20). The pUE constructs were also placed into *B. sphaericus* 718 and SSII-1 by protoplast transformation (19, 20).

Detection in immunoblots. Cells of *B. subtilis* DB104, *B. sphaericus* 718, *B. sphaericus* SSII-1, and *E. coli* JM105 which contained the appropriate recombinant plasmid produced 51- and 42-kDa crystal proteins detected in immunoblots with antibody to these proteins from *B. sphaericus* 2362 (19, 20). In all cases, more of the 42-kDa protein was produced than the 51-kDa protein. The 51-kDa protein was more stable than the 42-kDa protein, as indicated by the larger number of lower-molecular-weight degradation products derived from the 42-kDa protein. The smallest amount of protein was made in *E. coli* JM105, and more was made (in order of increasing amounts) in *B. sphaericus* SSII-1, *B. sphaericus* 718, and *B. subtilis* DB104. The small amount of protein made in *B. sphaericus* SSII-1 may be due to the poor sporulation of this strain.

Morphology of inclusions containing the 51- and 42-kDa proteins. Thin sections of sporulating cells containing pUE382 (which produces both the 51- and the 42-kDa proteins) were examined by the electron microscope. *B. subtilis* DB104 contained large amorphous inclusions, whereas *B. sphaericus* 718 and SSII-1 contained crystals indistinguishable from those of *B. sphaericus* 2362 (Fig. 5). These results suggest the presence of a factor(s) absent in *B. subtilis* necessary for ordered aggregation of the 51- and 42-kDa proteins. Such a factor must also be present in *B. thuringiensis*, since crystalline inclusions of the *B. sphaericus* toxin are formed in this species (16). It should be noted that in the case of lepidoptera-active toxins of *B. thuringiensis*, crystalline inclusions are produced by *B. subtilis* (6).

Larvicidal activity of bacterial cells containing the 51- and 42-kDa proteins. Table 2 gives the concentrations of various preparations, containing both the 51- and the 42-kDa proteins, necessary to kill 50% of the larvae of *Culex pipiens* (LC_{50}). The spore-cell-amorphous inclusion complex of *B. subtilis* DB104(pUE382) had an LC_{50} threefold lower than that of the *B. sphaericus* 2362 spore-cell-crystal complex.

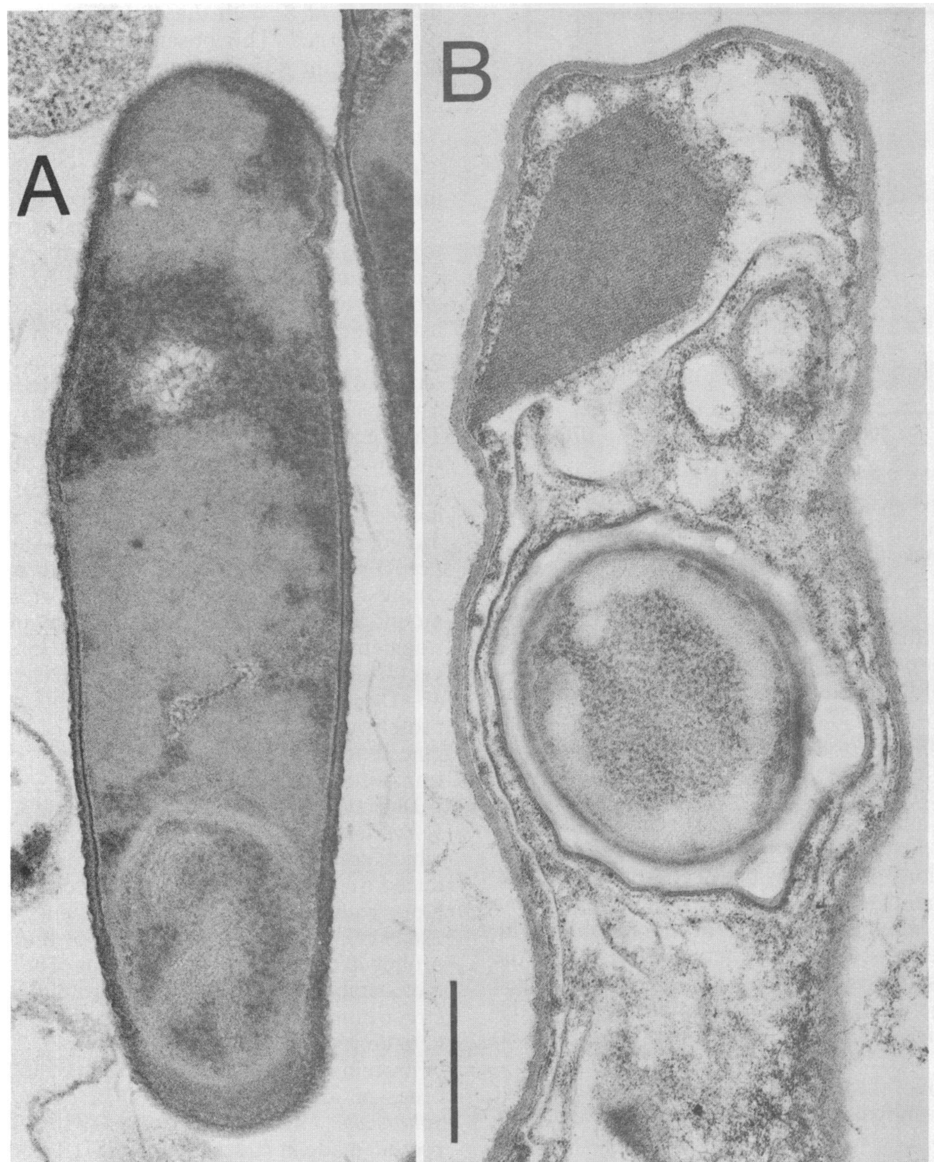

FIG. 5. Electron micrographs of (A) *B. subtilis* DB104 containing amorphous inclusions and (B) *B. sphaericus* 718 containing crystals. Both species harbor pUE382, a plasmid containing the genes for the 51- and 42-kDa proteins. Bar, 500 nm. Micrographs courtesy of Buzz Miller and Figen Seiler.

The LC_{50} of *B. sphaericus* 718 was similar to that of *B. sphaericus* 2362, whereas the LC_{50} of *B. sphaericus* SSII-1 was considerably higher. The highest value was that of cells of *E. coli* JM105. These results were in agreement with the amount of protein detected in immunoblots (19, 20). Cells of *B. subtilis* DB104 containing a construct which produced a fusion of the 51- and 42-kDa proteins had a toxicity comparable to cells in which both proteins were produced (19).

Evidence for binary toxin. Initial experiments used *E. coli* recombinants which produced small amounts of the 51- or the 42-kDa protein (9, 11). Neither of the cells was toxic alone; both were required for toxicity to mosquito larvae. These observations have been confirmed and extended by using cells which overproduce the 51- or the 42-kDa protein, indicating that the *B. sphaericus* larvicide is a binary toxin with respect to mosquito larvae. This property is not a function of the host cells in which the proteins are produced, as indicated by the requirement for the two proteins when the host cells are *E. coli* JM105, *B. subtilis* DB104, *B. sphaericus* 718, or *B. sphaericus* SSII-1 (19, 20). These observations cast doubt on the results of de la Torre et al. (46) which led to the conclusion that *B. subtilis* cells containing only the 42-kDa protein are toxic to mosquito larvae.

The ratio of the 51- to the 42-kDa protein necessary for maximal toxicity to mosquito larvae was determined by performing bioassays in which the relative amounts of amorphous inclusions containing each of the separate proteins were varied. The amounts of the 51- and 42-kDa proteins in the inclusions were determined by densitometry scans of Coomassie blue-stained gels (8). Approximately equal amounts of each protein were required for maximal toxicity. The LC_{50} at this optimal ratio (20 ng of protein per ml) is

TABLE 2. LC_{50}s of selected preparations containing both the 51- and 42-kDa proteins[a]

Host	Nature of inclusion	Assayed preparation	LC_{50}
B. sphaericus 2362	Crystalline	Spore-cell-crystal	18 ng(dry wt)/ml
		Crystal	7 ng of protein/ml
		NaOH-solubilized crystal	2,700 ng of protein/ml
		Purified 51- and 42-kDa proteins	12 ng of protein/ml
B. subtilis DB104(pUE382)	Amorphous	Spore-cell-amorphous inclusion	6 ng(dry wt)/ml
		Amorphous inclusion	11 ng of protein/ml
		NaOH-solubilized amorphous inclusion	920 ng of protein/ml
		Purified 51- and 42-kDa proteins	16 ng of protein/ml
B. sphaericus 718(pUE382)	Crystalline	Spore-cell-crystal	12 ng(dry wt)/ml
B. sphaericus SSII-1(pUE382)	Crystalline	Spore-cell-crystal	48 ng(dry wt)/ml
E. coli JM105(pKK382)	ND[b]	Cells	280 ng(dry wt)/ml

[a] Data compiled from references 8, 12, 19, and 20.
[b] ND, not determined.

similar to the LC_{50} obtained with amorphous inclusions containing both the 51- and the 42-kDa proteins (11 ng/ml [Table 2]).

In our first publication we reported that an electrophoretically homogeneous 42-kDa protein preparation from the crystal of *B. sphaericus* 2362 was toxic to *C. pipiens* (12). As our ability to obtain preparations containing fewer degradation products of the 51-kDa protein improved, the LC_{50}s of the 42-kDa protein preparations increased (20). The LC_{50}s were reduced by the addition of the 51-kDa protein. Recently, we purified a 42-kDa protein from *B. subtilis* DB104(pUE382) which produces both the 51- and the 42-kDa proteins (8). This purified protein was not toxic for mosquito larvae when tested alone up to a concentration of 200 μg of protein per ml. The LC_{50} of this protein in the presence of the 51-kDa protein was 21 ng of protein per ml. Another purified preparation of the 42-kDa protein from the crystal of *B. sphaericus* 2362 had an LC_{50} of 260 ng of protein per ml. The toxicity of this preparation was abolished by pretreatment with antiserum to the 51-kDa protein prior to its use in the bioassays (8). These results indicate that the toxicity of the "purified" 42-kDa protein preparation from *B. sphaericus* 2362 was due to contamination with degradation products of the 51-kDa protein and that the 42-kDa protein purified from this strain was not toxic alone (8, 12, 20, 23). The recent studies of Davidson et al. (38) also resulted in a similar conclusion.

Bioassays involving mosquito larvae. Bioassays involving mosquito larvae provide an estimate of the potential efficacy of a toxin preparation under natural conditions, since they test its effect on a living host. However, since the host is a complex organism with feeding preferences, the results obtained with different preparations frequently cannot be compared. Mosquito larvae are primarily particle feeders, although liquids are also consumed (3). There is a preference for particle size, which changes with the instar of the larva (31). Therefore, particulate preparations containing equal amounts of toxin may differ with respect to toxicity because of different particle sizes. Some of the difficulties with bioassays are illustrated in Table 2. The amorphous inclusions from *B. subtilis* DB104(pUE382) are slightly less toxic than the spore-cell-amorphous inclusion complex from this organism (11 and 6 ng/ml, respectively). This difference may be related to particle size. Solubilization of the crystal preparation from *B. sphaericus* 2362 resulted in a 386-fold increase in the LC_{50} (7 to 2,700 ng/ml), whereas solubilization of the amorphous inclusions from *B. subtilis* DB104 (pUE382) resulted in only an 84-fold increase in the LC_{50} (11 to 920 ng/ml). This suggests that the solubilized *B. sphaericus* preparation contains compounds which inhibit feeding. Highly purified soluble preparations from *B. sphaericus* crystals and *B. subtilis* amorphous inclusions containing approximately equal amounts of the 51- and 42-kDa proteins have nearly identical LC_{50}s for mosquito larvae, which are 225- and 58-fold lower, respectively, than the LC_{50}s of the original solubilized preparations (Table 2). A decrease of this magnitude in toxicity cannot be accounted for by the removal of contaminating proteins. The major differences between the particulate and solubilized preparations, as well as between the crude solubilized and purified soluble preparations, preclude the use of the larval assay as a means of accurately comparing toxin concentrations in different preparations or of monitoring recoveries during purification.

The midgut of mosquito larvae contains a variety of proteases and has a pH of about 10 (32). At this alkaline pH the crystal is solubilized and proteolytic processing or degradation occurs (24, 75). In mosquito bioassays used to detect the effects of deletions or other modifications of the mosquitocidal proteins, the change may have an effect on protein folding, thereby making the protein more accessible to the action of proteases found in the mosquito gut. The converse may also be a possibility: modified proteins may be renatured in the alkaline environment of the mosquito gut. When using bioassays to interpret the effects of protein modifications, it should be kept in mind that although the assay system has a major relevance in that the target is the living host, the lack of toxicity may be due to modifications of the proteins which only affect stability in the mosquito gut and do not affect sites in the protein essential for toxicity.

In Vivo Processing of Crystal Proteins

Following ingestion of the crystal of *B. sphaericus* 2362, *C. pipiens* larvae slowly degrade the 42-kDa protein to a 39-kDa derivative and rapidly degrade the 51-kDa protein (12). Similar results were obtained with solubilized crystal proteins treated with larval gut extracts containing proteases (12, 22). Analogous experiments with amorphous inclusions from *B. subtilis* containing the 42-kDa protein confirmed the accumulation of the 39-kDa derivative. Larvae which had ingested amorphous inclusions containing the 51-kDa protein accumulated a 43-kDa derivative (19). These results are summarized in Fig. 6. Similar conclusions were also obtained when using maxicells of *E. coli* containing radiola-

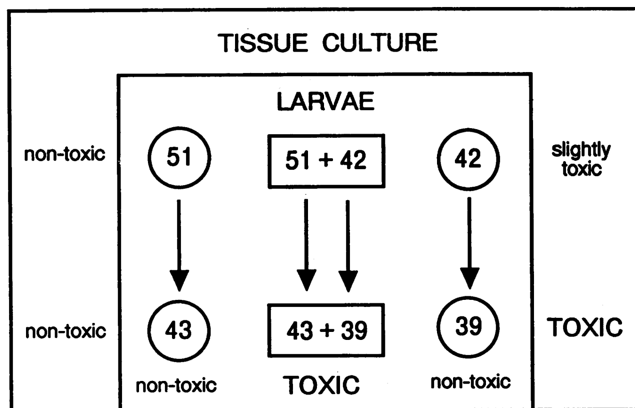

FIG. 6. Summary of the processing of the crystal proteins in mosquito larvae and toxicity of the proteins to larvae and mosquito tissue culture-grown cells. Arrows indicate proteolytic conversion of the toxin proteins in the larval gut.

beled 51- or 43-kDa protein or both (38). A fusion of the 51- and 42-kDa proteins was processed in vivo to proteins of 43 and 39 kDa, respectively (19).

Activation of the 42-kDa Protein

Bioassays involving tissue culture-grown cells. Since crystal proteins are processed to a lower molecular weight in the midgut, it is not possible to use larval bioassays to determine whether the higher-molecular-weight precursors are toxic. One solution to this problem is to use tissue culture-grown cells. The cell line most susceptible to the *B. sphaericus* toxin and most commonly used has been derived from ovarian tissues of *Culex quinquefasciatus* (53). Since the initial target site in vivo of the *B. sphaericus* toxin is the midgut epithelium, the results obtained with this cell line may differ in some aspects from those observed with larval midgut epithelial cells. The cultivation of cells in tissue culture may also lead to changes in surface receptors which affect susceptibility to toxins (48). Bioassays involving the *C. quinquefasciatus* cell line have used the trypan blue dye exclusion method or the determination of intracellular ATP by the firefly luciferin-luciferase assay as an indication of cell death (21, 34).

Processing of the 42-kDa protein results in activation. The observation that the 42-kDa protein is converted to a stable 39-kDa derivative in the gut suggested that, as is the case with several other toxins, proteolysis causes an activation or an increase in toxicity (6, 52). This was shown to be the case in subsequent studies (22, 37). The 42-kDa protein from *B. sphaericus* 2362 had an LC_{50} for tissue culture-grown cells of *C. quinquefasciatus* of 54 μg/ml (21). Purified 39-kDa protein derived from this 42-kDa protein by treatment with *C. pipiens* larval gut extracts had an LC_{50} of 1 μg/ml (22). Trypsin, chymotrypsin, and also protease-containing larval gut preparations from several other species of mosquitos converted the 42-kDa protein to a 39-kDa protein with the same increase in activity. Toxicity of the 39-kDa protein to tissue culture-grown cells as a result of contamination with a 51-kDa derivative was excluded by the fact that toxicity was not decreased by preincubation of the 39-kDa protein preparation with antiserum to the 51-kDa protein (8).

These results were confirmed by using recombinant-made 42-kDa protein and its chymotrypsin-activated 39-kDa product (8). The toxicity of the 39-kDa protein for tissue culture-grown cells of *C. quinquefasciatus* was not modified by the addition of the 51-kDa protein or a preparation containing its derivative, the 43-kDa protein (8). The results indicate that the 39-kDa protein has all of the determinants required for tissue culture toxicity. What, then, is the role of the 51-kDa protein or its derivative, the 43-kDa protein, in the toxicity to mosquito larvae but not to mosquito-derived tissue culture-grown cells? Since little is known concerning the mode of action of the toxin, it is not possible to frame speculations within the context of a mechanism. It has been shown that what is probably a mixture of the 42- and 51-kDa proteins or their derivatives binds to the cells of the gastric cecum and the posterior midgut as well as to tissue culture-grown cells of *C. quinquefasciatus* (36, 39). Recently, evidence has been presented indicating the same pattern of binding of the 51-kDa protein and the 42-kDa protein to the larval midgut (38). It seems doubtful that the mode of action on the target cells of the midgut differs from the mode of action on tissue culture-grown cells, since examination of both by means of electron microscopy shows a similar set of events (24, 40). For purposes of discussion we will assume that the 43-kDa derivative of the 51-kDa protein which accumulates in the gut of mosquito larvae (19) is the active form of this protein. It is possible that the 43-kDa protein has an accessory role in a productive attachment or penetration of the 39-kDa protein with respect to midgut target cells and that in the case of tissue culture-grown cells this function is not required. This difference could be due to a highly active internalization mechanism present in tissue culture-grown cells but absent from midgut epithelial cells. If this is the case, experiments with the cell line of *C. quinquefasciatus*, which is derived from ovarian tissue (53), has little or no relevance to the events occurring in the midgut cells. An alternative speculation is that the 43-kDa protein is essential in modifying some barrier (peritrophic membrane?) to the accessibility of the 39-kDa protein to the target cells and that this barrier is absent in tissue culture-grown cells.

Deletion Analysis of the 51- and 42-kDa Proteins

Deletions at the termini. By using site-directed mutagenesis, a series of deletions were constructed at the N and C termini of the 51- and 42-kDa proteins and their effect on toxicity for mosquito larvae was determined (23, 28). The goal of these studies was to establish the minimum size of the protein required for toxicity and to synthesize proteins equivalent to the processed derivatives which accumulate in the guts of mosquito larvae. The midgut of mosquito larvae contains primarily trypsinlike and some chymotrypsinlike activity (15, 61). Most of the constructed deletions at the N and C termini were made at potential chymotrypsin sites, whereas a few were made at potential trypsin sites. The choice of the deletions at chymotrypsin sites was based on the facts that (i) in the processing of the 42-kDa protein to the 39-kDa protein the cut at the N terminus was at a chymotrypsin site, (ii) digestion of the 42-kDa protein by larval gut proteases and by chymotrypsin results in 39-kDa products, and (iii) chymotrypsin sites are more evenly distributed at N and C termini than are trypsin sites, thereby allowing more uniform deletions. In our initial studies, constructs containing deletions of the 42-kDa protein were placed into pKK223-3 and overproduced in *E. coli* JM105. Immunoblots of proteins with deletions of 17 or more amino acids at the C terminus showed degradation to several fragments. The use of three other strains of *E. coli* containing mutations in

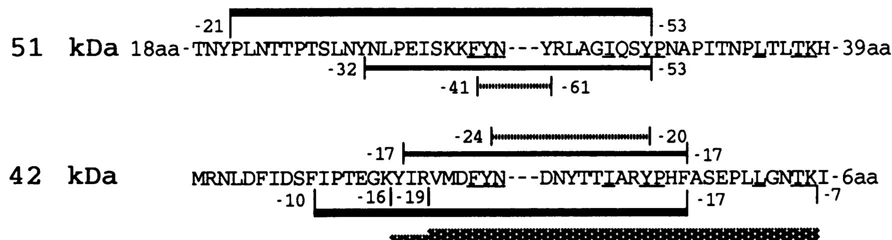

FIG. 7. Summary of the N- and C-terminal deletion analysis of the 51- and 42-kDa proteins expressed in *B. subtilis* DB104. Thick solid lines indicate proteins equivalent to those which accumulate in the larval gut and correspond to deletions at potential chymotrypsin sites. Thin solid lines indicate the smallest constructed protein which retained toxicity. Striped lines indicate the largest constructed protein with deletions at both termini which lacked toxicity. Numbers preceded by a negative sign indicate deletions from the N or C terminus. Deletions at the N terminus involve a change of the first remaining amino acid to methionine. The hatched lines at the bottom indicate the possible sizes of the trypsin-activated protein. Underlined residues are present in both the 51- and 42-kDa proteins. aa, amino acids.

functions affecting intracellular degradation did not significantly alter these results. The lack of toxicity for mosquito larvae of *E. coli* cells expressing 42-kDa derivatives with these deletions could therefore be due to their degradation. When the constructs were placed into the pUE vector and the proteins were overproduced in *B. subtilis* DB104, degradation was greatly reduced (23). Selected deletions were also expressed in *B. sphaericus* 718. Since the larvicide is a binary toxin, cells containing modified protein were tested in the presence of cells containing the intact coacting protein.

A summary of the studies involving pUE vectors and *B. subtilis* DB104 as the host (23, 28) is presented in Fig. 7. The 51-kDa protein derivative resulting from the deletion of 21 amino acids at the N terminus and 53 amino acids at the C terminus (−21,−53) has a calculated molecular mass of 43.1 kDa and corresponds to the 43-kDa protein which accumulates in the guts of mosquito larvae. A −10,−17 deletion of the 42-kDa protein results in a protein with a calculated molecular mass of 38.8 kDa and corresponds to the 39-kDa derivative which accumulates in larvae. Our studies indicate that a deletion between 33 and 41 amino acids at the N terminus of the 51-kDa protein and between 18 and 24 amino acids at the N terminus of the 42-kDa protein destroys toxicity. The −41 and −24 deletions at the N terminus of the 51- and 42-kDa proteins, respectively, which result in a loss of toxicity, involve the first two amino acids of the conserved sequence FYN (Fig. 7). These results suggest that the phenylalanine (F) and tyrosine (Y) of this sequence are required for toxicity and that perhaps this region of both proteins has a similar function. Similarly, a deletion of between 54 and 61 amino acids at the C terminus of the 51-kDa protein and between 18 and 20 amino acids at the C terminus of the 42-kDa protein also leads to a loss of toxicity. It would appear for the C terminus that the deletions leading to a loss of activity occur at sites of the 51- and 42-kDa proteins which have no amino acid sequence similarity.

Oei et al. (62) have performed a deletion analysis of the 51- and 42-kDa proteins from *B. sphaericus* 2297. The proteins were overproduced in *E. coli*, and the cells were used for bioassays. The sizes of the proteins were determined by radiolabeling the toxins in maxicells followed by performing electrophoresis of the cell extracts and autoradiography. With one exception, their results are in agreement with the results of our studies on deletions of the 51- and 42-kDa proteins of *B. sphaericus* 2362. Oei et al. (62) found that a 7-amino-acid deletion at the C terminus of the 42-kDa protein led to a loss of toxicity. We have found that a protein containing this deletion is toxic when overproduced in *E. coli* JM105 or *B. subtilis* DB104 (23). Since the electrophoretic mobility of the deleted protein of Oei et al. (62) suggests a higher molecular weight than the original protein and since the same deletion constructed by us migrated as expected, it would appear that the lack of toxicity of the deletion constructed by Oei et al. (62) was due to other modifications of the protein. Using *E. coli* as the host, Sebo et al. (66) showed that a derivative of the 42-kDa protein containing a −15 N-terminal deletion and a −17 C-terminal deletion was still toxic for mosquito larvae in the presence of the 51-kDa protein.

Trypsin digestion of the 42-kDa protein derived from *B. sphaericus* 2362 leads to an activated protein with an apparent molecular mass of about 39 kDa (22). Such a protein would correspond to either a −16,−7 or a −19,−7 derivative of the 42-kDa protein; these derivatives have calculated molecular masses of 39.2 and 38.8 kDa, respectively. These results indicate that there is some flexibility with respect to the cuts necessary for activation of the 42-kDa protein.

Hybrid proteins, internal deletions, and duplications. The existence of regions having a similar amino acid sequence in the 51- and 42-kDa proteins (Fig. 4 and 8) suggests that they may be essential for toxicity and that portions of the 51- and 42-kDa proteins may be functionally equivalent. The latter possibility was tested by constructing a series of hybrids of the 51- and 42-kDa proteins and testing the cells containing these products for toxicity alone and in combinations with cells containing the reciprocal hybrid and original proteins (29). For example, the hybrid protein ABc (Fig. 8) was tested for toxicity (i) alone, (ii) with abC, (iii) with abC and ABC, and (iv) with abC and abc. None of the combinations had

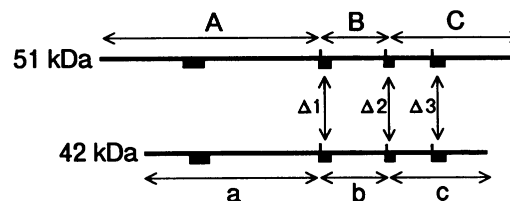

FIG. 8. Diagram showing the fragments used for the construction of hybrids and deletions of the 51- and 42-kDa proteins. Capital letters designate fragments of the 51-kDa protein, and lowercase letters designate fragments of the 42-kDa protein. Thick lines represent regions with a conserved amino acid sequence. Δ1, Δ2, and Δ3 indicate deletions of 12, 6, and 16 amino acids, respectively.

toxicity to mosquito larvae, thereby indicating that the original combination of fragments is unique in that the fragments are not exchangeable. This result also suggests that the two proteins differ in at least one functional attribute. That the conserved regions 1, 2, and 3 are essential for toxicity to mosquito larvae is indicated by the fact that a deletion of any one of these sequences abolishes toxicity. Proteins containing an internal duplication of the regions B and b (ABBC and abbc) had a greatly reduced toxicity when tested together or with the coacting original protein (29).

MODE OF ACTION AND HOST SPECIFICITY OF TOXIN

The most extensive study, by means of the electron microscope, of the changes in the larval midgut epithelium following ingestion of sporulated *B. sphaericus* cells is that of Charles (24). Confirming and extending previous observations (74, 75), Charles showed that in the highly susceptible species *C. pipiens*, the primary changes occur in the cells of the gastric cecum and the posterior midgut, with the appearance of areas of low electron density, vacuolation, and mitochondrial swelling. In contrast to the effect observed with *B. thuringiensis* subsp. *israelensis* (25), there does not appear to be a general dissolution of the midgut cells. Binding in the gastric cecum and the posterior midgut was detected with fluorescein-labeled toxin (35, 36) and *E. coli* maxicells containing radiolabeled 51- or 42-kDa protein (38). There was evidence of internalization of the fluorescein-labeled toxin into cells of the posterior midgut (35).

A number of studies have established that the action of the crystal toxin on susceptible larvae involves the following series of steps: (i) ingestion of the crystal-spore-cell complex; (ii) solubilization in the midgut by the alkaline pH; (iii) processing of the 51- and 42-kDa proteins to 43- and 39-kDa proteins, respectively; (iv) binding of toxin proteins to cells of the gastric cecum and posterior midgut; and (v) exertion of a toxic effect by means of an unknown mechanism. Different species of mosquito larvae vary greatly in their susceptibility to *B. sphaericus* crystal toxins. It is instructive to make a comparison of these steps in the highly susceptible species *C. pipiens* and the resistant species *Aedes aegypti*. *A. aegypti* ingests *B. sphaericus* at a rate slightly higher than that of *C. pipiens* (4). Electron-microscopic observation indicates that the crystal is dissolved in the guts of both larvae (24). Proteases from both species are able to activate the 42-kDa protein by digestion, and the resulting 39-kDa protein has the same toxicity (22). The only significant difference between these species appears to be the binding of the toxin proteins to the gastric cecum and posterior midgut of *C. pipiens* but not *A. aegypti* (35, 36). Steps (i) to (iii) have also been demonstrated in the larvae of other mosquito species (24, 35, 36, 42, 61). These results suggest that differences in susceptibility of mosquito larvae to the toxin are in part due to differences in the number of binding or target sites. This interpretation is also supported by the results of studies involving tissue culture-grown cells from mosquito species which vary greatly in their susceptibility (22, 34, 61).

OTHER BINARY TOXINS

The most extensively studied binary toxins are the anthrax toxin (69) and the C2 botulinum toxin (1). Both toxins consist of two proteins, one of which is involved in binding to the cell membrane, whereas the other performs a toxic function. The C2 toxin activity is due to ADP-ribosylation of actin, whereas the mode of action of the lethal component of the anthrax toxin is unknown. Both proteins of the anthrax toxin have been cloned and sequenced; neither of them has any sequence similarity with each other or with the *B. sphaericus* toxin (18, 71). The components of the C2 botulinum toxin have been shown to be antigenically distinct; neither has been cloned and sequenced, so a comparison with the *B. sphaericus* toxins cannot be made. It is possible that the conservation of sequences between the 51- and 42-kDa proteins is an indication of a shared function. If this is the case, the *B. sphaericus* toxins would be different from the binary toxins of anthrax and botulinum C2, which consist of functionally distinct subunits.

CONCLUSIONS AND FUTURE PROSPECTS

The 51- and 42-kDa mosquitocidal crystal proteins of *B. sphaericus* are unique among bacterial insect toxins in that they (i) act as a binary toxin when tested against mosquito larvae, (ii) have a low sequence similarity, and (iii) are distinct from all of the cloned and sequenced insect toxins of *B. thuringiensis*. The present review indicates that the characterization of these toxins by the techniques of genetic engineering and molecular biology is nearly complete. What is left is the much more difficult task of understanding the function of the components of the binary toxin, their interaction with cells, and the mechanism of the toxic action.

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