BACILLUS SPHAERICUS
TOXINS: Molecular Biology and Mode of Action

J.-F. Charles, C. Nielsen-LeRoux, and A. Delécluse
Bactéries Entomopathogènes, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

KEY WORDS: crystal toxin, Mtx toxins, toxin genes, receptor, resistance

ABSTRACT
Bacillus sphaericus is a spore-forming aerobic bacterium, several strains of which are pathogenic for mosquito larvae. During sporulation, the most active strains produce a crystal toxin with a high degree of larvicidal activity. The toxin is composed of two proteins of 51.4 and 41.9 kDa, which are encoded by highly conserved chromosomal genes. After B. sphaericus is ingested, these proteins are released in the larva's midgut, and, in susceptible mosquito species, bind to a specific receptor present on midgut brush-border membranes. The resulting damages to the midgut cells leads to the mosquitoes' death. During vegetative growth, some B. sphaericus strains also synthesize mosquito larvicidal proteins of 100 and 30.8 kDa (Mtx toxins), the mode of action of which is still unknown. The mechanism of acquisition of the recessive mosquito resistance to the crystal toxin varies with selection conditions.

PERSPECTIVES AND OVERVIEW
The use of microorganisms as a source of biological compounds for insect pest control started after the discovery of the highly insecticidal bacteria Bacillus thuringiensis. The discovery of the strain B. thuringiensis serovar israelensis (35, 45) made possible efficient microbiological control of Diptera Nematocera vectors of diseases, such as mosquitoes (Culicidae) and black flies (Simuliidae).

The first reported Bacillus sphaericus strain active against mosquito larvae was isolated from moribund mosquito larvae (51). The larvicidal activity of this isolate was so low that their use in mosquito control would never have been considered (Table 1). The identification of the strain SSII-1 in India (73)
renewed the search for more active strains. But only after the isolation (in Indonesia from dead mosquito larvae) of strain 1593—which exhibited a much higher level of mosquitocidal activity (74) was the potential of *B. sphaericus* as a biological-control agent for mosquitoes taken seriously (75).

*B. sphaericus* (57) is an aerobic bacterium that produces terminal spherical spores. It lacks several biochemical pathways and thus cannot use sugars as metabolites. The findings of various genetic and biochemical studies indicate that this species is heterogenous. The increasing number of isolated strains has made differentiating between toxic and atoxic strains, and between the toxic strains themselves, difficult.

Numerous methods have been used to classify this very heterogenic species. For example, relationships between strains of *B. sphaericus* have been examined in terms of DNA homology (53). Based on the percentage of homology, five groups were identified, and group II was further subdivided into IIA (related by over 79% homology and containing all toxic strains) and IIB. Classification via two other systems, bacteriophage typing (85, 87) and serotyping using flagellar antigens (36, 38), yielded a similar grouping for the group IIA mosquito pathogenic strains. Currently, nine serotypes (listed in Table 1) are known to contain active strains. Recently developed techniques, such as numerical classification based on taxonomy of phenotypic features (2, 46), cellular fatty acid composition analysis (42), ribotyping (3), and random amplified polymorphic DNA analysis (84), indicate that most of the pathogenic strains are recovered in a few groups.

None of these methods allows us to predict the level of toxicity of a given strain. The three mosquitocidal activity levels do not correspond to the groups defined by any other method of classification (Table 1). The genes encoding various toxins have been cloned (see below), and they might be used in hybridization experiments to predict activity (4). However, some strains react positively with toxin genes but display only weak larvicidal activity. Therefore, to identify potentially valuable strains and to further elucidate the nature of the mosquito-larvicidal activity, we must continue to determine toxicity levels by using mosquito larvae.

*B. sphaericus* strains are generally highly active against larvae of *Anopheles* and *Culex* species and are poorly or not toxic to larvae of *Aedes* species. However, susceptibility appears to depend on the species of mosquito and can thus vary within a genus (14). Toxicity levels vary among the larvicidal serotypes and even within the same serotype (82). Therefore, the relative potency of each strain is currently evaluated and described in terms of specific activity titers on the different mosquito species and in terms of activity ratios derived from such titers (82).

Like *B. thuringiensis* against Lepidoptera, Singer (73) first suggested that *B. sphaericus* acts by toxemia rather than septicemia. A few years later,
### Table 1  Comparative properties of some mosquitocidal strains of B. sphaericus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Source</th>
<th>Flagellar serotype</th>
<th>Phage type</th>
<th>DNA group</th>
<th>RNA (R) group</th>
<th>Fatty acid group</th>
<th>Mosquitocidal activity$^b$</th>
<th>Crystal genes</th>
<th>Mtx genes</th>
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<td>L</td>
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<td>+ NA</td>
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<td>RIIA</td>
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<td>−</td>
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<td>M</td>
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<td>− −</td>
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<td>−</td>
<td>I</td>
<td>RI</td>
<td>AIV</td>
<td>−</td>
<td>−</td>
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$^a$ Data compiled from References 2, 3, 84, 42, 46, 53, 80, 87; I Thiéry, AA Yousten, A Porter, and C Berry (personal communications); and from the IEBC (Institut Pasteur). +, present; −, absent; NA, no information available.

$^b$ Based on the lethal concentration 50% after 48 h on fourth-instar larvae of Culex pipiens ($LC_{50}$): L, $≥ 1 × 10^{-3}$ sporulated culture dilution; M, $LC_{50} = 1 × 10^{-4}$ dilution; H, $LC_{50} = 1 × 10^{-6}$ dilution.

$^c$ Strains not responding to any of the bacteriophages tested.

$^d$ B. sphaericus type strain.
Davidson & Myers (28) reported the presence of parasporal inclusions, which they suspected participate in the toxic action of \textit{B. sphaericus}. Indeed, all of the most toxic strains produce parasporal inclusions during sporulation. These inclusions have a crystalline ultrastructure and are released into the medium along with the spore after the completion of sporulation (Figure 1). The relationship between sporulation, crystal formation, and mosquitocidal activity was first clearly established for strains belonging to serotypes H5a5b (18, 56), H25 (37, 49, 86), and H6 (39). Later, the partial purification of crystals toxic to mosquito larvae (64), and the study of mutants blocked at early stages of sporulation that fail to make crystals and are not mosquitocidal (24), confirmed the toxic nature and protein composition of the crystals. More recently, the identification of a group of toxins different from the crystal toxins, the Mtx toxins, renewed interest in \textit{B. sphaericus} as a mosquitocidal agent (80, 81).

Two other recent reviews discuss the genetics of \textit{B. sphaericus} toxins and their mode of action (11, 66). The increasing application of \textit{B. sphaericus} in the field has recently led to cases of resistance. To restrict the development of resistance, we must understand the nature and mode of action of \textit{B. sphaericus} toxins, as well as the mechanism of resistance. These issues have been extensively investigated, and an overview of each is given below.
BIOCHEMISTRY AND GENETICS OF *B. SPHAERICUS* TOXINS

Two kinds of toxins (crystal and Mtx toxins) seem to account for the larvicidal activity of *B. sphaericus*. They differ both in their composition and time of synthesis. The crystal toxins are present in all highly active strains and are produced during sporulation. The Mtx toxins are responsible for the activity of most of the weakly active strains. Mtx proteins seem to be synthesized only during the vegetative phase.

**Crystal Toxins**

The crystal toxin is composed of two proteins that are synthesized in equimolar amounts and assembled in crystal structures visible at about stage III of sporulation (12, 49, 86). The proteins are designated as P51 and P42 on the basis of their predicted molecular masses of 51.4 and 41.9 kDa (5, 9, 10, 13, 15, 47, 48). The genes encoding both proteins have been cloned from several high-toxicity strains. They appear to be organized in an operon with a 174- to 176-base pair (bp) intergenic region. A stem-loop structure (characteristic of transcription terminators) lies downstream from the P42 gene (Figure 2a) (9, 47). No sequence similar to *Bacillus subtilis* sporulation promoters has been found upstream from the gene encoding P51. However, both the P42 and P51

![Diagram of B. sphaericus toxins](image)

**Figure 2** Genes encoding the *B. sphaericus* toxins (left) and schematic representation of corresponding polypeptides (right). (a) Crystal toxin. The homologous regions shared by P51 and P42 are represented by identical patterns. (b) Mtx toxins. The black boxes indicate the potential signal sequences; the diagonally striped box denotes the potential transmembrane sequence; the two shaded regions are homologous with ADP-ribosyltransferase toxins; and the crosshatched box signifies internal repeated sequences. Arrows point to the tryptic cleavage sites determined for each toxin. Potential ribosome binding sites and terminator sequences are also indicated for each toxin.
genes are expressed only during sporulation in *B. subtilis* (7). Moreover, *lacZ* fusions to the promoter of the crystal protein genes indicate that, in *B. sphaericus*, transcription begins immediately before the end of exponential growth and continues into stationary phase (1). Thus, enough protein for crystal formation accumulates before stage III.

The genes for P51 and P42 are chromosomal, at least in strains 1593, 2362, and 2297, although these strains contain plasmids (3). Both genes have been reported in diverse strains, and all highly toxic strains tested contain similar sequences as assessed using DNA hybridization (4). The reverse is not true: Moderately toxic strains such as LP1-G also contain these genes (Table 1). The toxin-coding and flanking regions of strains 1593, 2362, and 2317-3 are identical over a span of 3479 nucleotides, whereas the sequences in strains IAB59 and 2297 differ by 7 and 25 nucleotides, respectively (15). The resulting differences in the amino acid sequences of P51 are 5 and 3 amino acids, respectively and those for P42 are 1 and 5. These variations appear to be responsible for the differences in specificity of these strains (see below). Interestingly, LP1-G is only weakly active, although it produces the crystal toxin (Table 1). The sequence of crystal-toxin genes from this strain may indicate which regions of the protein are involved in the loss of toxicity.

The amino acid sequences of P51 and P42 are not similar to those of any other bacterial toxins, including those produced by *B. thuringiensis* serovar *israelensis* (Bti). However, P51 and P42 share four segments of sequence similarity (9) (Figure 2), the significance of which remains unclear. Therefore, P51 and P42 of *B. sphaericus* constitute a separate family of insecticidal toxins (9).

The aggregation of both P51 and P42 has been analyzed using crystal-toxin components expressed separately or together in homologous or heterologous *Bacillus* hosts. When expressed independently in *B. subtilis* and *B. sphaericus* or in *B. thuringiensis* crystal-negative hosts, the proteins form amorphous inclusions (23, 59); in contrast, crystals similar to those produced by naturally occurring, highly toxic *B. sphaericus* strains are produced when the two genes are simultaneously expressed in either *B. sphaericus* or *B. thuringiensis* (23, 59). No crystal can be detected in *B. subtilis* even when both genes are present, unless the genes are fused, eliminating the intergenic region (23). These results suggest that *B. sphaericus* and *B. thuringiensis* contain factors that help stabilize the proteins and subsequent crystallization of the toxin.

In vivo, P42 is slowly converted into a stable protein of ~39 kDa, whereas P51 is rapidly converted into a stable fragment of ~43 kDa (12, 19). In vitro deletion analysis led to the delineation of the minimal active fragments of both proteins, which indeed correspond to the activated fragments (20, 26, 62, 70). At the N and C terminus of P51, 32 and 53 amino acids, respectively, can be eliminated without loss of toxicity (26); deletions of 10 and 17 amino acids at
the N and C terminus of P42, respectively, result in a protein similar to the 39-kDa activated fragment (20) (Figure 2a).

Subcloning experiments have shown that P42 alone is toxic for mosquito larvae (*Culex pipiens pipiens*), although the activity is weaker than that of crystals containing both proteins (59). In contrast, P51 alone is not toxic, but its presence enhances the larvicidal activity of P42, suggesting synergy between the polypeptides (17, 40, 59, 62). In vitro assays on mosquito cell lines also revealed that the activated form of P42 alone is toxic to *Culex pipiens quinquefasciatus* cells, whereas P51 appears to be inactive; however, no synergy between the components was observed in vitro (8). Although simultaneous presence of both proteins appears necessary for full toxicity, the activity toward various mosquito species displayed by the different *B. sphaericus* strains depends on the origin of P42, as shown by analysis of toxins mutated in vitro (14). For example, when amino acids were substituted in a region centered around position 100 of P42, the IAB59 and 2297 P42 proteins became similar to the protein from strain 2362: The activity and specificity of the mutant toxins toward *Culex* and *Aedes* larvae were comparable, unlike those of the wild-type. This finding indicates that this region is involved in specificity. Together, these observations suggest that P42 is the most important determinant of specificity and activity.

**Mtx Toxins**

Two types of Mtx toxins have been described to date: Mtx and Mtx2, with molecular masses of 100 and 30.8 kDa, respectively; genes for both these toxins have been cloned from the medium-toxicity strain SSII-1 (80, 81). In contrast to the crystal toxin genes, *mtx* and *mtx2* genes are expressed during the vegetative growth phase, and sequences resembling the vegetative promoters of *B. subtilis* have been found upstream from each gene (80, 81). Fusions of the *lacZ* gene to the *mtx* promoter also confirmed vegetative expression, because β-galactosidase activity was restricted to the early exponential phase, at least in *B. sphaericus* (1). Both proteins possess short N-terminal leader sequences, which is characteristic of gram-positive bacterial signal peptides (80, 81). However, both toxins have been found associated with the cell membrane of *B. sphaericus*, which indicates little or no cleavage of the signal sequence. The Mtx toxin can be further processed by gut proteases into two fragments of 27 and 70 kDa that correspond to the N- and C-terminal regions, respectively (79). The 70-kDa fragment from Mtx possesses three repeated regions of ~90 amino acids each (Figure 2b), the function of which is unknown; the 27-kDa fragment contains a short region corresponding to a transmembrane domain. The Mtx and Mtx2 toxins do not display any similarity to each other or to the crystal proteins or any other insecticidal proteins. However, the
27-kDa part of Mtx shares weak similarity with the catalytic domain of several ADP-ribosylating toxins (Figure 2b) (79, 80). Mtx2 has 34 and 29% similarity to two toxins active against mammalian cells, namely the ε-toxin from *Clostridium perfringens* and the cytotoxin from *Pseudomonas aeruginosa*, respectively (81).

The genes encoding Mtx and Mtx2 are widespread among various *B. sphaericus* strains (55, 81), including those with low, medium, and high toxicity (Table 1). The toxicity of Mtx has been measured using fusion proteins synthesized in *Escherichia coli*: It is highly active against mosquito larvae (79), with an LC₅₀ value comparable to that of the two-component crystal proteins (15 ng protein/ml). Deletion analysis suggests that the 27-kDa fragment from Mtx can ADP-ribosylate itself, whereas the 70-kDa fragment is responsible for toxicity to cultured *C. pipiens quinquefasciatus* cells; however, both regions are necessary for toxicity to mosquito larvae (78).

**MODE OF ACTION OF THE CRYSTAL TOXIN**

The mode of action of *B. sphaericus* crystal toxin has only been studied in mosquito larvae. However, in one report, the toxin was active in adults of *C. pipiens quinquefasciatus*, but not in *A. aegypti* adults, after introduction by enema into the midgut (77).

After ingestion of the spore-crystal complex by mosquito larvae, the protein-crystal matrix quickly dissolves in the lumen of the anterior stomach (21, 25, 86) through the combined action of midgut proteinases and the high pH (22, 27). *B. sphaericus* crystals release the toxin in all species, even in non-susceptible species such as *A. aegypti* (Figure 1c). Indeed, some studies report that the differences in susceptibility to *B. sphaericus* between mosquito species do not result from differences in solubilization and/or activation of the crystal toxin (12, 31, 58).

**Cytopathology and Physiological Effects**

*Bi* toxins completely break down the larval midgut epithelium, whereas *B. sphaericus* toxins do not. Nevertheless, midgut alterations start as soon as 15 min after ingestion of the *B. sphaericus* spore-crystal complex (21, 28, 50, 76). Midgut damages in *Culex pipiens* are the same after ingestion of spore crystals of either strain 1593 or strain 2297 (21, 28, 76). In contrast, the symptoms of intoxication produced by these two strains differ in other mosquito species. Large vacuoles (and/or cytolyzosomes) appear in *C. pipiens* midgut cells, whereas large areas of low electron density appear in *Anopheles stephensi* midgut cells (compare Figures 3b and 3c with the control, Figure 3a). A generally occurring symptom is mitochondrial swelling, described for *C. pipi-
Figure 3  (a) Midgut cell of untreated *A. stephensi* larva.  (b) Midgut cell of *A. stephensi* after feeding on *B. sphaericus* 2297.  (c) Midgut cell of *C. pipiens pipiens* after feeding on *B. sphaericus* 2297.  N, nucleus, PM, peritrophic membrane, V, vacuoles.  Arrows indicate cytolysosomes; stars indicate areas of low electron density.

*ens pipiens* and *A. stephensi*, as well as for *A. aegypti* when intoxicated with a very high dose of spore crystals (21).  The midgut cells, especially those of the posterior stomach and the gastric caeca, are the cells most severely damaged by the toxin, and Singh & Gill (76) also report late damage in neural tissue and in skeletal muscles.

Ultrastructural effects have been reported in cultured cells of *C. pipiens quinquefasciatus* within a few minutes of treatment with soluble and activated *B. sphaericus* toxin.  These alterations consisted mainly of swelling of mitochondrial cristae and endoplasmic reticula, followed by enlargement of vacuoles and condensation of the mitochondrial matrix (33).

The physiological effects of *B. sphaericus* crystal toxin have been poorly
documented. Lakshmi Narasu & Gopinathan (54) report that oxygen uptake by mitochondria isolated from B. sphaericus–treated C. pipiens quinquefasciatus larvae is inhibited, as is the activity of larval choline acetyl transferase in the presence of toxin. In addition, B. sphaericus toxin decreases oxygen uptake by mitochondria isolated from rat liver (54).

**Binding to a Specific Receptor in the Brush-Border Membrane**

As the differences in susceptibility between mosquito species do not seem related to the ability to solubilize and/or activate the binary toxin, this variation presumably results from differences at the cellular level. Indeed, studies report the binding of fluorescently labeled toxin to the gastric caeca and the posterior stomach only in very susceptible Culex species. P51 does not bind to the gut of A. aegypti, whereas P42 binds weakly and nonspecifically in this species. No regional binding was observed in Anopheles spp. (29, 30, 34, 63). Furthermore, these studies showed that, in C. pipiens quinquefasciatus, only P51 binds specifically to the caecum and posterior stomach, whereas P42 binds nonspecifically throughout the midgut (Figure 4). When the proteins are together, the binding of P42 appears to depend on the binding of P51. In vivo binding studies using nontoxic deletion mutants of the crystal toxin (63) support the hypotheses that the N-terminal region of P51 is involved in regional binding of this protein in the larval midgut and that the C-terminal region of P51 and the N- and C-terminal regions of P42 are involved in the interaction of the two components that causes P42 to bind in the same regions as P51. These authors also report that internalization of toxin only seems to occur when both components are present. However, further intracellular investigations are required to elucidate whether one or both components are internalized.

The hypothesis that a specific receptor was involved in the toxin binding was confirmed by in vitro binding assays using $^{125}$I-labeled activated crystal toxin and midgut brush-border membrane fractions (BBMFs) isolated from either susceptible or nonsusceptible mosquito larvae (60). Indeed, direct binding experiments with C. pipiens pipiens BBMFs indicated that the toxin binds to a single class of specific receptor. The toxin-receptor binding characteristics include a dissociation constant ($K_d$) of $20 \pm 5$ nM toxin and a receptor concentration ($R$) of $7 \pm 4$ pmol toxin/mg of BBMF protein (Figure 5a). No significant specific binding was detected with BBMFs from A. aegypti (Figure 5b), which was consistent with the lack of specific binding in fluorescence-labeling studies (30, 32). Both crystal toxin components were bound to the membranes of the susceptible species, but the linearity of the Scatchard representation clearly confirmed that only one of them bound to a receptor (Figure 5a, inset).

However, binding studies exposing radiolabeled P42 and P51 separately to
Figure 4  (a) Midgut of C. pipiens quinquefasciatus larva fed with fluorescently labeled P42. This protein binds over the entire midgut. (b) Larva fed with fluorescently labeled P42 and unlabeled P51; binding of P42 is regional, restricted to the gastric caeca and posterior stomach. AS, anterior stomach, GC, gastric caeca, PS, posterior stomach. Micrographs kindly provided by Dr. C Berry, Department of Biochemistry, University of Wales, College of Cardiff.

BBMF might give valuable information about toxin-receptor interactions and the kinetics for each component. If we assume that the P42 component is the toxic (active) moiety (59, 70) and P51 is the binding component, the B. sphaericus crystal toxin is more likely to be similar to an A/B toxin than to a binary toxin. The nature of the receptor is still unknown. Sugars including N-acetylamino-D-galactose or N-acetylamino-D-glucose do not inhibit the binding of the toxin to the receptor (60). Therefore, these sugar moieties are probably not involved in the recognition and binding of the toxin in C. pipiens pipiens larvae.

INSECT RESISTANCE TO B. SPHAERICUS CRYSTAL TOXIN

The risk of emergence of resistance should be considered when designing application strategies. Furthermore, an understanding of the mechanism of resistance might also lend insight into the mode of action of the toxin.
Five cases of \textit{C. pipiens} larval resistance to \textit{B. sphaericus} crystal toxin have been reported (Table 2) in both laboratory-selected populations (43, 69) and field populations after treatment with \textit{B. sphaericus} (67, 71, 72). The mechanism of resistance to \textit{B. sphaericus} crystal toxin has been studied extensively in only two \textit{C. pipiens quinquefasciatus} populations: a highly resistant laboratory-selected population (43) and a field-treated population with a low level of resistance (71).

**Laboratory-Selected Resistance**

A highly resistant \textit{C. pipiens quinquefasciatus} (\textit{CpqR}) population was obtained under intense selection pressure by exposing a large number of early fourth-instar larvae in 12 successive generations to \textit{B. sphaericus} toxin under a high selection pressure (43; GP Georghiou et al, unpublished data). Tests of the toxicity of \textit{B. sphaericus} SPH88 (strain 2362) against susceptible (\textit{CpqS}) and resistant (\textit{CpqR}) larvae, their F\textsubscript{1} progeny (\textit{CpqS} × \textit{CpqR}), and the backcross (BC) offspring (\textit{F\textsubscript{1}} × \textit{CpqR}) showed that the \text{LC}_{50} of the resistant strain was more than 100,000-fold higher than that of the susceptible strain, and that their dose-response slopes were equal. Likewise, the susceptibility of the \textit{F\textsubscript{1}} was
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<tbody>
<tr>
<td>C. pipiens quinquefasciatus USA (California)</td>
<td>Strain 2362</td>
<td>In the laboratory (field colony)</td>
<td>100,000</td>
<td>1.500 – 2,500</td>
<td>F₁₂</td>
<td>LC₀₉₀-₉₀</td>
<td>Lab: yes</td>
<td>10,000 – 20,000</td>
</tr>
<tr>
<td>C. pipiens quinquefasciatus USA (California)</td>
<td>Strain 2362</td>
<td>In the laboratory (field colony)</td>
<td>27</td>
<td>1,500 – 2,500</td>
<td>F₀₉₀</td>
<td>LC₀₉₀-₉₀</td>
<td>Lab: yes</td>
<td>10,000 – 20,000</td>
</tr>
<tr>
<td>C. pipiens quinquefasciatus India (Kochi)</td>
<td>Biocide® strain 1595M</td>
<td>In the laboratory (field colony)</td>
<td>6,200</td>
<td>ND</td>
<td>F₀₉₀</td>
<td>LC₀₉₀-₉₀</td>
<td>Field: no</td>
<td>150</td>
</tr>
<tr>
<td>C. pipiens quinquefasciatus Brazil (Recife)</td>
<td>Spherimos® strain 2362</td>
<td>In the field</td>
<td>57 treatments</td>
<td>2 years</td>
<td>F₀₉₀</td>
<td>LC₀₉₀-₉₀</td>
<td>Field: no</td>
<td>37 treatments</td>
</tr>
<tr>
<td>C. pipiens pipiens France (Port Saint-Louis)</td>
<td>Spherimos® strain 2362</td>
<td>In the field</td>
<td>10</td>
<td>2 years</td>
<td>F₀₉₀</td>
<td>LC₀₉₀-₉₀</td>
<td>Field: no</td>
<td>37 treatments</td>
</tr>
</tbody>
</table>

*Data compiled from References 41, 61, 67, 71, 72. G. Sinégre and N. Pasteur (personal communications).

Ratio of the LC₉₀ of the resistant population to the LC₉₀ of the susceptible population.
very close to that of the susceptible parent, indicating recessive resistance. The susceptibility of BC larvae showed no linear relationship to toxin concentration.

Binding experiments (61) showed that all available receptors in the parental CpqS population were saturable (Figure 6a). The corresponding Scatchard plot was linear, indicating that the radiolabeled toxin binds to a single class of specific binding sites, as previously found for the B. sphaericus receptor from *C. pipiens pipiens* (60). In the CpqR population, the toxin concentrations used did not result in specific binding to the BBMFs, whereas the nonspecific binding was equivalent to the one occurring in CpqS (Figure 6b). The BBMFs from the F1 progeny had a single class of binding sites (Figure 6c) with characteristics similar to those of the susceptible strain. The binding sites of BBMFs from the BC progeny, CpqBC, were not saturated by toxins in the range of concentrations used, and the total amount of bound toxin was much lower than for the CpqS strain (Figure 6d). The LIGAND computer program analysis (55a) of the binding data obtained with the BC progeny suggests that the experimental data fit a two-site model better than a one-site model.

The results of binding experiments with labeled *B. sphaericus* binary toxin agreed with bioassay data. The resistance is recessive, and the two classes of binding sites found in the BC populations suggest genetic heterogeneity.

The extremely high resistance level to *B. sphaericus* that results after intense selection pressure (43) does not appear under lower selection pressure (69). This finding might indicate that the selection pressure, the number of individuals treated per generation, and the gene frequency of the resistant alleles are important elements in the potential development of high-level resistance by the mosquito populations.

**Field-Selected Resistance**

The efficacy of *B. sphaericus* treatment for control of the *C. pipiens quinquefasciatus*, which is the vector for Bancroftian filariasis, was tested for two years in Recife (Brazil). Good control of the vector was obtained, but the susceptibility of the field-treated population to *B. sphaericus* decreased significantly (68). Bioassays indicated that the resistance level was 6.5-fold after 33 treatments and 10.2 fold after 37 treatments (71). In vitro binding experiments using radiolabeled *B. sphaericus* toxin and BBMFs from the treated or control larval populations indicated no change in the affinity for the toxin of the midgut receptor of the treated population (*K_d* = 11 nM). Only one class of binding sites was found in the two populations, and only a slight decrease in receptor concentration was observed in the resistant population (*R_t* = 7.5 from 9.2 pmol/mg). Thus, the mechanism of resistance involved in the field-selected resistance did not appear to involve receptor functionality.
Figure 6  Binding of $^{125}$I-labeled *B. sphaericus* binary toxin to *C. pipiens quinquefasciatus* midgut brush-border membranes from (a) susceptible (*CpqS*), (b) resistant (*CpqR*), (c) cross $S \times R$ (*CpqF1*), and (d) backcross $F_1 \times R$ (*CpqBC*) populations.
The resistance levels of 150-fold reported from India (67) and 16,000-fold from France (72) clearly show that high levels of resistance can emerge under field conditions. In both regions, treatments were repeated, generation turnover was high, and mixing with nonselected populations was low.

Recent results from in vitro binding studies using BBMFs from the *B. sphaericus*–resistant *C. pipiens pipiens* population from France suggest that the mechanism of resistance does not involve a receptor modification at least in its binding function (C Nielsen-LeRoux, unpublished data). Therefore, the mechanism of field resistance requires further investigation.

**Physiological Function of the Receptor**

Because resistance is stable in the absence of selection pressure and the resistant population appears to have good fitness in the wild, mutation of the receptor molecule probably does not suppress the expression of the receptor's normal physiological function. Alternatively, the selected individuals may possess another molecule with the same function. If the toxin receptor on the membrane has an important function in the cell, as does the aminopeptidase-N that is the receptor for *B. thuringiensis* CryIA(c) in the tobacco hornworm, *Manduca sexta* (52), the modification of one receptor could be compensated for by other molecules that have a similar physiological function. This hypothesis is supported by the observation that field populations of *B. sphaericus*–resistant *C. pipiens quinquefasciatus* in Brazil and India were more susceptible than the normal populations to Bti (two- and sevenfold, respectively) (67, 71). Experiments have shown that labeled *B. sphaericus* toxin and nonlabeled toxins from Bti crystal proteins do not compete for binding sites (C Nielsen-LeRoux, unpublished data), indicating that they have different receptors. An increase in the synthesis of other molecules, such as those able to bind Bti toxins, may compensate for the loss of *B. sphaericus* susceptibility. The high-level laboratory-selected *B. sphaericus*–resistant *C. pipiens quinquefasciatus* population was as susceptible to Bti as the unselected population; this observation suggests that laboratory- and field-selected resistance involve different mechanisms.

**CONCLUSIONS**

The sequences of the genes encoding P42 and P51, and of the regions important for the specificity and activity of the components, have been almost elucidated. Both P42 and P51 are needed for full activity of the *B. sphaericus* crystal toxin: P42 alone is weakly toxic, and P51 appears to be responsible for the regional binding of the toxin to a highly specific and saturable midgut membrane receptor. The nature of the receptor, the intracellular interactions, and
the target molecules of the crystal toxin remain unclear, and further work is required to elucidate the mode of action. The Mtx toxins have been less extensively studied than the crystal toxins, and their modes of action probably differ.

Despite the reports of resistance, the future of *B. sphaericus* in the control of mosquito larvae is promising. Indeed, resistance in the field seems to decline very quickly when treatments are suspended (R Reuben & L Regis, personal communication), probably because of recessive gene expression. G Sinègre (personal communication) found resistance in only one breeding site in an area treated for eight years (two or three treatments a year). In addition, cross resistance between strains of *B. sphaericus* harboring different sequences has not been found to occur (43), which indicates that the different toxins may bind to different receptors. Baseline susceptibility information on target mosquito populations should be collected before treatment is started, and their susceptibility should be monitored continually to detect any significant shifts in baseline toxicity. Treatment should then be stopped before the resistance becomes a problem. Georghiou et al (44) showed that the natural variation in the susceptibility of *C. pipiens quinquefasciatus* to *B. sphaericus* was much higher than the variation in susceptibility to *Bti*. Thus, the probability of selecting for resistance to *B. sphaericus* is probably higher than that for *Bti*.

The use of *B. sphaericus* strains belonging to different serotypes and displaying no cross resistance (43) could minimize the risk of resistance. This alternative requires a search for new strains with crystal toxins whose amino acid sequences vary from that deduced for the commonly used strain, 2362. This approach seems promising, as strains such as 2297 and IAB59 produce crystal toxins different from the 2362 toxin (see section on biochemistry and genetics of the toxins). Novel crystal toxins could also be produced by construction of mutant toxins. Mutation experiments performed by Berry et al (14) suggest that this is also a feasible approach.

The best way to prevent resistance may be to produce bacterial strains that simultaneously express different toxins binding to different receptors. In addition to the crystal toxins, other toxins from *B. sphaericus* or other insecticidal microorganisms could be produced. The Mtx toxins, which do not seem to be expressed at the same time as the crystal toxins, are potential alternatives; expression of Mtx genes during sporulation of *B. sphaericus*, i.e. under the control of a sporulation promoter, could allow diversification of toxins. Similarly, *Bti* mosquitocidal toxin genes can be combined with *B. sphaericus* genes; such efforts include the introduction of the genes encoding CryIVB or CryIVD from *Bti* into toxic *B. sphaericus* strains (6, 65, 83). Conversely, *B. sphaericus* crystal toxin genes have been introduced into toxic *Bti* (16). Although these studies did not demonstrate any increase in toxicity against *Anopheles* and *Culex* species, such recombinants may delay insect resistance and should be
studied in this regard. Several screening programs have now been established to isolate novel mosquitocidal toxins that could be combined with both Bti and B. sphaericus toxins. New toxins identified from one Clostridium bifermentans strain and several B. thuringiensis strains appear to be good candidates for use in combination with B. sphaericus toxins (41). The challenge now is to express stably and simultaneously these toxin genes in the same host and to study the development of resistance.

ACKNOWLEDGMENTS

We are very grateful to all our colleagues who helped us prepare this review by providing unpublished data, micrographs, and/or helpful comments, especially Drs. C Berry, University of Wales; G Georghiou and MS Mulla, University of California, Riverside; A Porter, National University of Singapore; F Priest, Heriot-Watt University; L Regis, Fiocruz, Recife; R Reuben, CRME, Madurai; and AA Yousten, Virginia Polytechnic Institute. We are also indebted to Dr. I Thiéry and V Patncio (Institut Pasteur) for mosquito rearing, S Hamon (Institut Pasteur) for mosquito bioassays, H Ohayon and C Rollin (Institut Pasteur) for technical assistance, and A Edelman for critical reading of the manuscript. A large part of our work has been supported by the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases, and by grants from the Commission of the European Communities SCIENCE Program, from the Danish International Development Assistance, from the University of California Mosquito Research Program, and from the CAPES and the CNPq (Brazil).

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