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Cytopathological Effects of *Bacillus sphaericus* Cry48Aa/Cry49Aa Toxin on Binary Toxin-Susceptible and -Resistant *Culex quinquefasciatus* Larvae[▽]

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The Cry48Aa/Cry49Aa mosquitocidal two-component toxin was recently characterized from *Bacillus sphaericus* strain IAB59 and is uniquely composed of a three-domain Cry protein toxin (Cry48Aa) and a binary (Bin) toxin-like protein (Cry49Aa). Its mode of action has not been elucidated, but a remarkable feature of this protein is the high toxicity against species from the *Culex* complex, besides its capacity to overcome *Culex* resistance to the Bin toxin, the major insecticidal factor in *B. sphaericus*-based larvicides. The goal of this work was to investigate the ultrastructural effects of Cry48Aa/Cry49Aa on midgut cells of Bin-toxin-susceptible and -resistant *Culex quinquefasciatus* larvae. The major cytopathological effects observed after Cry48Aa/Cry49Aa treatment were intense mitochondrial vacuolation, breakdown of endoplasmic reticulum, production of cytoplasmic vacuoles, and microvillus disruption. These effects were similar in Bin-toxin-susceptible and -resistant larvae and demonstrated that Cry48Aa/Cry49Aa toxin interacts with and displays toxic effects on cells lacking receptors for the Bin toxin, while *B. sphaericus* IAB59-resistant larvae did not show mortality after treatment with Cry48Aa/Cry49Aa toxin. The cytopathological alterations in Bin-toxin-resistant larvae provoked by Cry48Aa/Cry49Aa treatment were similar to those observed when larvae were exposed to a synergistic mixture of Bin/Cry11Aa toxins. Such effects seemed to result from a combined action of Cry-like and Bin-like toxins. The complex effects caused by Cry48Aa/Cry49Aa provide evidence for the potential of these toxins as active ingredients of a new generation of biolarvicides that conjugate insecticidal factors with distinct sites of action, in order to manage mosquito resistance.

Bacillus sphaericus is considered an important entomopathogen due to its capacity to produce insecticidal proteins with specific action against mosquitoes (Diptera: Culicidae). The binary (Bin) toxin, which is produced during bacterial sporulation and deposited in parasporal crystalline inclusions, is the most important larvicidal factor. Other proteins characterized, such as mosquitocidal toxins (Mtx proteins), can be produced during vegetative growth, and although these proteins may have larvicidal potential, they play a minor role in the toxicity of the native strains since they are produced by vegetative cells and are degraded by *B. sphaericus* proteinases (20, 30), and do not form components of the spore-crystal preparations that are used in control programs. Recently, a new two-component toxin was characterized from *B. sphaericus* strain IAB59. This is formed by the proteins Cry48Aa (135 kDa) and Cry49Aa (53 kDa), which are produced as crystalline inclusions (13). The toxin has a unique composition since the Cry48Aa component belongs to the three-domain family of Cry proteins with 30% similarity to the mosquitocidal Cry4Aa protein from *Bacillus thuringiensis* serovar israelensis, while Cry49Aa is one of the Bin-toxin-like proteins, a family that comprises the Bin toxin from *B. sphaericus*, in addition to the Cry36 and Cry35 proteins from *B. thuringiensis* (9, 13).

Cry48Aa/Cry49Aa is considered a two-component toxin because neither component shows toxicity alone, whereas both can act in synergy and the optimum level of toxicity to *Culex* species is achieved when the two are present at an equimolar ratio. The 50% lethal concentration for third-instar larvae equates to 15.9 ng/ml Cry48Aa and 6.3 ng/ml Cry49Aa of purified toxins, which is a level of toxicity comparable to that of the Bin toxin (13). However, in contrast to the Bin toxin, which is naturally produced in an equimolar ratio, Cry48Aa production is low in native strains and does not confer high toxicity (13). The initial steps of the mode of action of Bin and Cry48Aa/Cry49Aa crystals are similar and comprise the ingestion of crystals, solubilization under alkaline pH, and activation of protoxins into toxins by midgut proteases. After processing, Bin toxin recognizes and binds to specific receptors in the midgut of Bin-toxin-susceptible species through its subunit BinB (51 kDa), while the component BinA (42 kDa) confers toxicity and is likely to form pores in the cell membrane (7, 25). The membrane-bound receptors of Bin toxin on the midgut of *Culex quinquefasciatus* larvae, Cqm1, were characterized as 60-kDa α-glucosidases (24). The mode of action of Cry48Aa/Cry49Aa is still unknown, but a remarkable feature of this new two-component toxin is the capacity to overcome *C. quinquefasciatus* resistance to the Bin toxin (13, 19, 21). Resistance of

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Culex larvae to the Bin-toxin-based larvicides often relies on the absence of functional Cqm1 receptors in the midgut (19, 24, 26). As a consequence, toxins with a distinct mode of action, such as Cry48Aa/Cry49Aa as well as *B. thuringiensis* serovar israelensis toxins (Cry11Aa, Cry4Aa, Cry4Ba, and Cyt1Aa), do not experience cross-resistance in the Bin-toxin-resistant larvae (12, 21, 32). Such toxins can play a strategic role in the management of resistance, and the major goal of this study was to investigate the ultrastructural effects of the Cry48Aa/Cry49Aa toxin on Bin-toxin-susceptible and -resistant *C. quinquefasciatus* larvae and to compare these with the effects of a synergistic mixture of Bin/Cry11Aa used to overcome Bin toxin resistance.

MATERIALS AND METHODS

***Culex quinquefasciatus* colonies.** Fourth-instar larvae from three colonies were used in this study. CqSF is the reference colony susceptible to all *B. sphaericus* toxins; CqRL1/2362 is a laboratory-selected colony highly resistant (>100,000-fold) to Bin toxin and susceptible to the IAB59 strain (21); CqRL2/IAB59 is a laboratory-selected colony highly resistant (≈40,000-fold) to *B. sphaericus* IAB59 (1). All colonies have been maintained in the insectarium of the Centro de Pesquisas Aggeu Magalhães-FIOCRUZ for more than 5 years under controlled conditions of 26 ± 1°C, 70% relative humidity, and a 12-h/12-h (light/dark) photoperiod. Larvae were reared in dechlorinated water and fed on cat food. Adults were maintained on a 10% sugar solution, and females were also fed on chickens.

Recombinant toxins. Cry48Aa and Cry49Aa toxins, recently characterized in the *B. sphaericus* strain IAB59 (13), were produced individually in the acrystalliferous *B. thuringiensis* serovar israelensis strain 4Q7, transformed with the plasmids pSTAB135 and pHTP49 carrying the genes encoding those toxins, respectively (13). Bin toxin from *B. sphaericus* strain 1593 and Cry11Aa toxin from *B. thuringiensis* serovar israelensis were produced in strain 4Q7, transformed with the plasmids pGSP10 (3) and pHT640 containing the respective genes encoding those proteins. Cultures were grown using a sporulation medium (17) supplemented with 1% glucose and erythromycin (25 μg/ml), or tetracycline for the plasmid pGSP10, under agitation at 30°C, until reaching sporulation (>80%). Spore-crystal biomass was recovered by centrifugation, washed with 1 M NaCl-10 mM EDTA and with 10 mM EDTA sequentially, and stored at −20°C. Later, the biomass containing spore-crystals from each toxin was lyophilized and 25 mg of powder samples was solubilized in 50 mM NaOH, under agitation at 30°C for 1 h. Supernatants containing solubilized proteins were separated by centrifugation at 17,000 × *g*, at 4°C for 30 min. Protein concentration was determined by Bio-Rad assay using a bovine serum albumin standard curve, and samples were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cry48Aa (135-kDa) and Cry49Aa (53-kDa) lyophilized powders yielded 12.6 and 6.2 mg/ml of in vitro-solubilized proteins, respectively. Powders containing spore-crystals from each protein were used in bioassays, as described, in a 1:1 (wt/wt) mixture to give a toxin ratio of Cry48Aa/Cry49Aa which was close to equimolarity, according to protein contents of each component. Bin/Cry11Aa was also used as a mixture of lyophilized powders at a 3:1 (wt/wt) ratio.

In vivo toxicity assays. Bioassays were performed according to the standard method recommended by the World Health Organization (31). Groups of 20 early-fourth-instar larvae placed in 100 ml of distilled water in plastic cups were used in the assays. Bacterial suspensions at 5 g/liter of each powder mixture, Cry48Aa/Cry49Aa and Bin/Cry11Aa, were prepared. Larvae from CqSF and CqRL1/2362 colonies were treated with final concentrations of 10 and 20 mg/liter, respectively, of the Cry48Aa/Cry49Aa mixture at 1:1 (wt/wt) to provoke approximately 90% larval mortality after 48 h of exposure, while larvae from the CqRL2/IAB59 colony were treated with 30 mg/liter. Larvae from the CqRL1/2362 colony were also treated with a mixture of Bin/Cry11Aa at 7.5 mg/liter, a level able to provide high larval mortality. A control group tested with water only was run in each experiment. All larvae were fed with a small amount of food during the assays. Samples of larvae from treated and untreated groups were collected and processed for transmission electron microscopy, as described below. The remaining larvae from the experimental groups were kept to record larval mortality after a 48-h exposure period.

Dissection and processing for transmission electron microscopy. To analyze the ultrastructural effects of Cry48Aa/Cry49Aa and Bin/Cry11Aa on midgut epithelial cells of *C. quinquefasciatus*, early-fourth-instar larvae were dissected 1 and 6 h after being exposed to the bacterial suspensions. Ten larvae from each experimental group were chilled on ice, and the midguts were excised under a binocular microscope. After removal of the food enclosed by the peritrophic membrane and the Malpighian tubules, the posterior midgut was processed for transmission electron microscopy analysis. Specimens were fixed overnight at 4°C, with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. After fixation, samples were washed twice in the same buffer and postfixed in 1% osmium tetroxide, 5 mM $CaCl_2$, and 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2. Samples were then dehydrated in acetone and embedded in Epon 812 resin (Sigma, St. Louis, MO). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 transmission electron microscope.

TABLE 1. Mortality of *Culex quinquefasciatus* fourth-instar larvae from a Bin-toxin-susceptible colony (CqSF), a Bin-toxin-resistant colony (CqRL1/2362), and a *Bacillus sphaericus* IAB59-resistant colony (CqRL2/IAB59), after a 48-h treatment with spore-crystal mixtures of Cry48Aa/Cry49Aa and Bin/Cry11Aa, produced individually as recombinant proteins

Colony	Cry48Aa/Cry49Aa, 1:1 (wt/wt)[a]			Bin/Cry11Aa, 3:1 (wt/wt)		
	Toxin concn (mg/liter)	No. of larvae	Mortality (%)	Toxin concn (mg/liter)	No. of larvae	Mortality (%)
CqSF	10	140	94.2			
CqRL1/2362	20	40	100	7.5	80	81.3
CqRL2/IAB59	30	80	3.8			

[a] Cry48Aa and Cry49Aa lyophilized powders yielded 12.6 and 6.2 mg/ml of in vitro-solubilized proteins, respectively.

RESULTS

The effects of Cry48Aa/Cry49Aa toxin were investigated on larvae from three *C. quinquefasciatus* colonies: Bin-toxin-susceptible larvae (CqSF), Bin-toxin-resistant larvae (CqRL1/2362), and *B. sphaericus* IAB59-resistant larvae (CqRL2/IAB59). Treated groups of CqSF and CqRL1/2362 larvae showed over 90% mortality after 48 h of exposure, compared to less than 4% mortality recorded for CqRL2/IAB59 resistant larvae (Table 1). Untreated groups of larvae did not display significant mortality levels (data not shown). The activity of Cry48Aa/Cry49Aa toxin against Bin-toxin-susceptible CqSF and CqRL1/2362 larvae was thus confirmed, while larvae selected for *B. sphaericus* IAB59 resistance were not susceptible to these components.

The ultrastructural analysis of midgut from untreated CqSF larvae showed cells with a preserved aspect characterized by numerous packed microvilli and abundant mitochondria and rough endoplasmic reticulum (Fig. 1A). Cells from treated larvae showed mitochondrial alterations after 1 h of treatment. These organelles became swollen and vacuolated and showed disorganized mitochondrial cristae, without disruption of the outer membrane (Fig. 1B). The presence of small vesicles in the cytoplasm, probably derived from the endoplasmic reticulum rupture, and the appearance of electron-dense granules and vacuoles were observed (Fig. 1C). Most cells from 1-h-treated larvae presented well-preserved microvilli (Fig. 1D); however, disruption of this structure was observed in larvae after 6 h of treatment (Fig. 1E). Swollen and vacuolated mitochondria (Fig. 1E) and cytoplasmic vacuoles of increased

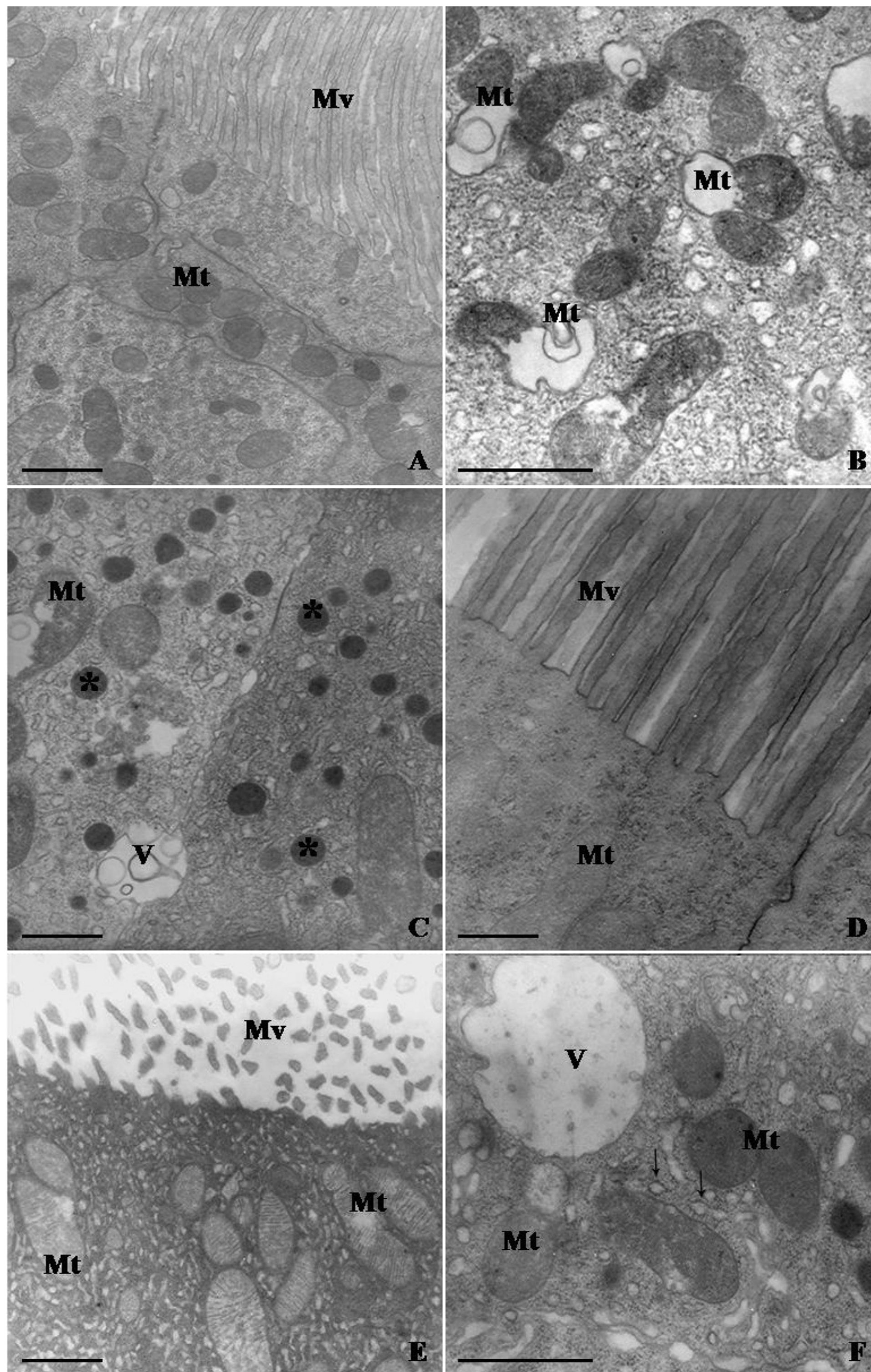

FIG. 1. Transverse ultrathin sections of the posterior midgut from fourth-instar *Culex quinquefasciatus* Bin-toxin-susceptible larvae (CqSF) treated with Cry48Aa/Cry49Aa toxin from *Bacillus sphaericus* IAB59. (A) Cell from a nontreated larva rich in microvilli (Mv) and mitochondria (Mt). (B) Cell from 1-h-treated larvae showing mitochondrial vacuolation (Mt). (C) Cell from 1-h-treated larvae presenting electron-dense granules (*), cytoplasmic vacuoles (V), and small vesicles from endoplasmic reticulum breakdown. Mt, mitochondria. (D) Cell from 1-h-treated larvae with preserved microvilli (Mv) and mitochondrial swelling (Mt) at the apical side of the cell. (E) Cell from 6-h-treated larvae showing evident microvillus damage (Mv) and mitochondrial vacuolation (Mt). (F) Cell from 6-h-treated larvae presenting large cytoplasmic vacuoles (V) and small ribosome-coated vesicles (arrows). Mt, mitochondria. Bars, 1 μm.

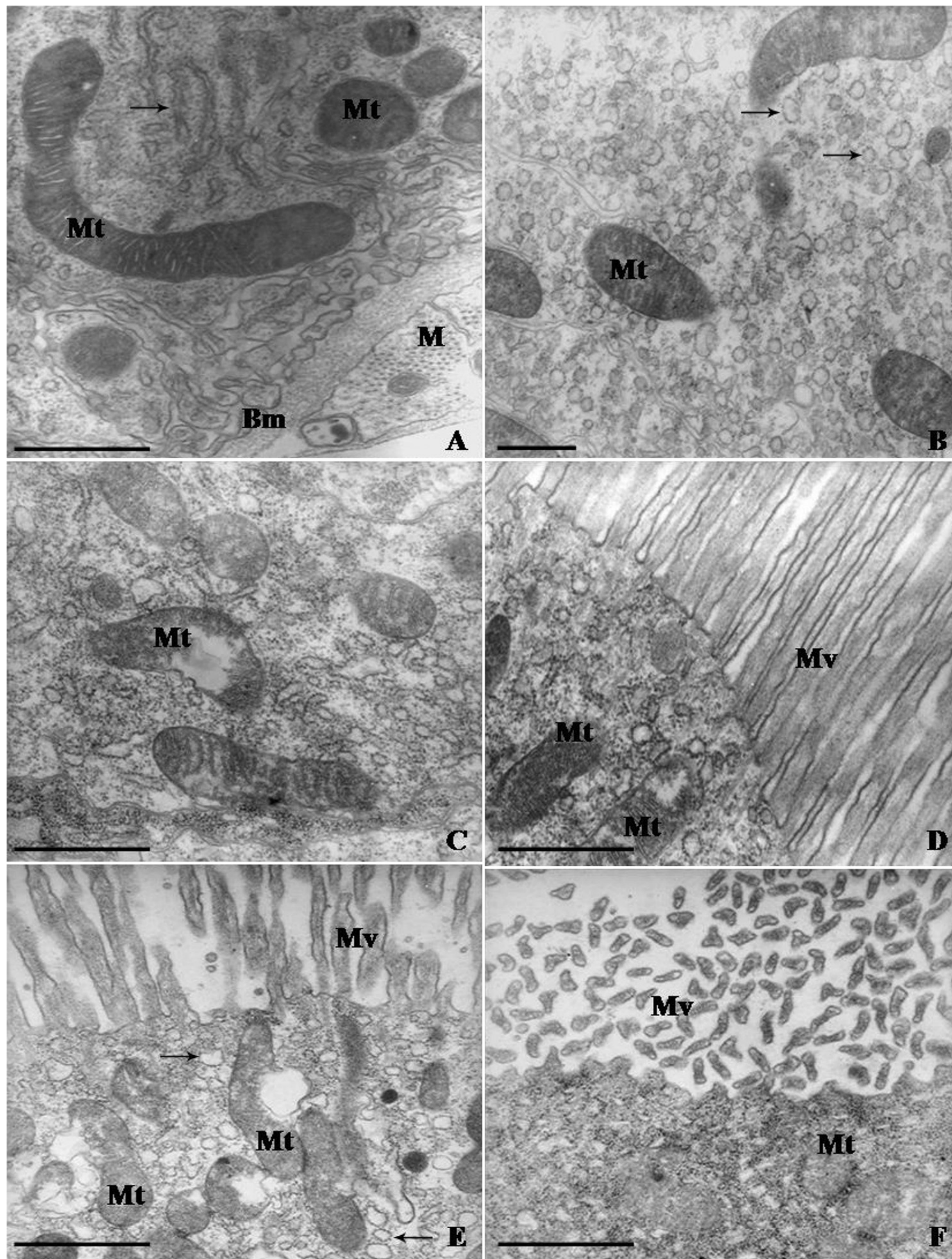

FIG. 2. Transverse ultrathin sections of the posterior midgut from fourth-instar *Culex quinquefasciatus* larvae resistant to the Bin toxin (CqRL1/2362) treated with Cry48Aa/Cry49Aa toxin from *Bacillus sphaericus* IAB59. (A) Cell from a nontreated larva with preserved organelles such as mitochondria (Mt) and endoplasmic reticulum (arrow); the underlying basal membrane (Bm) and myoid cell (M) also exhibited a normal appearance. (B) Cell from 1-h-treated larvae showing mitochondrial matrix destruction (Mt) and the appearance of ribosome-coated vesicles (arrows). (C) Cell from 1-h-treated larvae showing mitochondrial vacuolation (Mt) without external membrane breakdown. (D) Cell from 1-h-treated larvae presenting preserved microvilli (Mv) and mitochondria (Mt) in the process of vacuolating. (E) Cell from a 6-h-treated larva with mitochondrial vacuolation (Mt), endoplasmic reticulum breakdown (arrows), and microvillus rupture (Mv). (F) Cell from a 6-h-treated larva showing huge microvillus destruction (Mv) and cytoplasm disorganization. Bars, 1 μm.

size (Fig. 1F) were also observed in cells from 6-h-treated larvae.

Midgut cells from CqRL1/2362 untreated larvae showed an appearance similar to that described previously (Fig. 2A; compare to Fig. 1A). Larvae from this colony were susceptible to Cry48Aa/Cry49Aa (Table 1) and showed morphological cell alterations after treatment, similar to those of CqSF larvae. The major effects observed after 1 h of treatment were the rupture of the endoplasmic reticulum, resulting in resealed small vesicles throughout the cytoplasm (Fig. 2B and C), and mitochondrial vacuolation, without disruption of the outer membrane (Fig. 2C and D). CqRL1/2362 larvae presented the same pattern of microvillus alterations as did CqSF larvae, in which the effects were observed only 6 h after treatment (Fig.

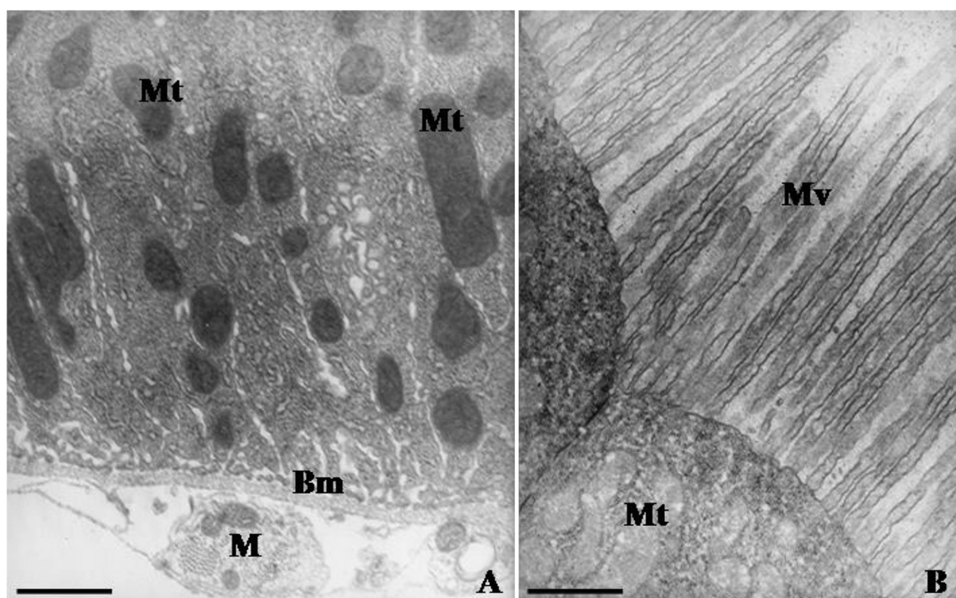

FIG. 3. Transverse ultrathin sections of the posterior midgut from fourth-instar *Culex quinquefasciatus* larvae resistant to *Bacillus sphaericus* IAB59 (CqRL2/IAB59), treated with Cry48Aa/Cry49Aa toxin from this strain. (A) Basal side of epithelial cell from 6-h-treated larvae presenting mitochondria (Mt) without vacuolation and underlying basal membrane (Bm) and myoid cell (M) that are well preserved. (B) Cell under the same treatment conditions showing preserved microvilli (Mv). Mt, mitochondria. Bar, 1 μm.

2E and F), subsequent to mitochondrial vacuolation and endoplasmic reticulum disruption (Fig. 2E).

Larvae from the CqRL2/IAB59 colony, which are resistant to all the toxins produced by *B. sphaericus* strain IAB59 (including Bin and Cry48Aa/Cry49Aa), presented only discrete alterations such as the development of electron-dense granules after 1 or 6 h of exposure to Cry48Aa/Cry49Aa (data not shown). Microvilli showed a preserved appearance, and mitochondria were swollen but did not present vacuolation (Fig. 3A and B).

The combination of Bin/Cry11Aa toxins was used to characterize morphological alterations caused by synergistic action between these toxins on Bin-toxin-resistant larvae, as well as for comparison with the effects of Cry48Aa/Cry49Aa toxin. Treatment with Bin/Cry11Aa produced close to 80% mortality of CqRL1/2362 larvae after 48 h (Table 1), while the nontreated groups did not show significant mortality (data not shown). Midgut epithelial cells of untreated CqRL1/2362 larvae showed a morphological appearance similar to that described before for CqSF larvae (Fig. 4A). After 1 h of treatment with Bin/Cry11Aa toxins, the mitochondria from cells of CqRL1/2362 larvae showed mitochondrial crista destruction and vacuolation (Fig. 4B), similar to the effects of Cry48Aa/Cry49Aa toxin on midgut cells of CqSF and CqRL1/2362 larvae. The cytoplasm became dense after 1 h of treatment, and mitochondria in the epithelial cell showed disorganized cristae compared with the mitochondria from the myoid cell, located below the basal membrane, which also showed a well-preserved layer of muscular fibers (Fig. 4C). After 6 h of treatment, the cytoplasmic and mitochondrial matrix showed a dense aspect, while the basal membrane was intact (Fig. 4D). Large cytoplasmic vacuoles containing cellular debris and vacuolated mitochondria were detected (Fig. 4E), in a pattern similar to that observed in CqSF larvae treated with Cry48Aa/Cry49Aa toxin, 6 h after exposure. In contrast, disrupted microvilli were not observed in the cells of CqRL1/2362 larvae after Bin/Cry11Aa treatment (Fig. 4F).

DISCUSSION

The morphological effects of the Cry48Aa/Cry49Aa two-component toxin from *B. sphaericus* on midgut epithelial cells of *C. quinquefasciatus* larvae are described in this work. Major alterations observed after Cry48Aa/Cry49Aa exposure include cytoplasmic vacuolation, fragmentation of endoplasmic reticulum, and microvillus disruption. Similar effects have been also described after Bin toxin treatment (5, 8, 10, 15, 27); however, mitochondrial vacuolation, characterized by internal matrix destruction and deformation of the organelle without rupture of the external membrane, was a singular effect distinct from those recorded previously for the Bin toxin (10). Injuries to mitochondria were important effects associated with Cry48Aa/Cry49Aa treatment. Dysfunctions of these organelles can be critical since they lead to an increase of reactive oxygen species that cause damage to major cellular components (2). Such changes in mitochondrial morphology (mitochondrial swelling and inner matrix destruction) have previously been reported for the action of *B. thuringiensis* toxins on midgut epithelia of mosquitoes and lepidopteran larvae (4, 6, 16, 18, 23). The ultrastructural effects of Cry48Aa/Cry49Aa thus appear to be a combination of those of Bin and three-domain Cry toxins. A similar effect was seen when the mixture of Bin toxin from *B. sphaericus* and Cry11Aa from *B. thuringiensis* serovar israelensis was used to overcome Bin toxin resistance in CqRL1/2362 larvae, which was comparable to the effect caused by Cry48Aa/Cry49Aa. With the Bin/Cry11Aa toxin combination, the major effect observed was mitochondrial

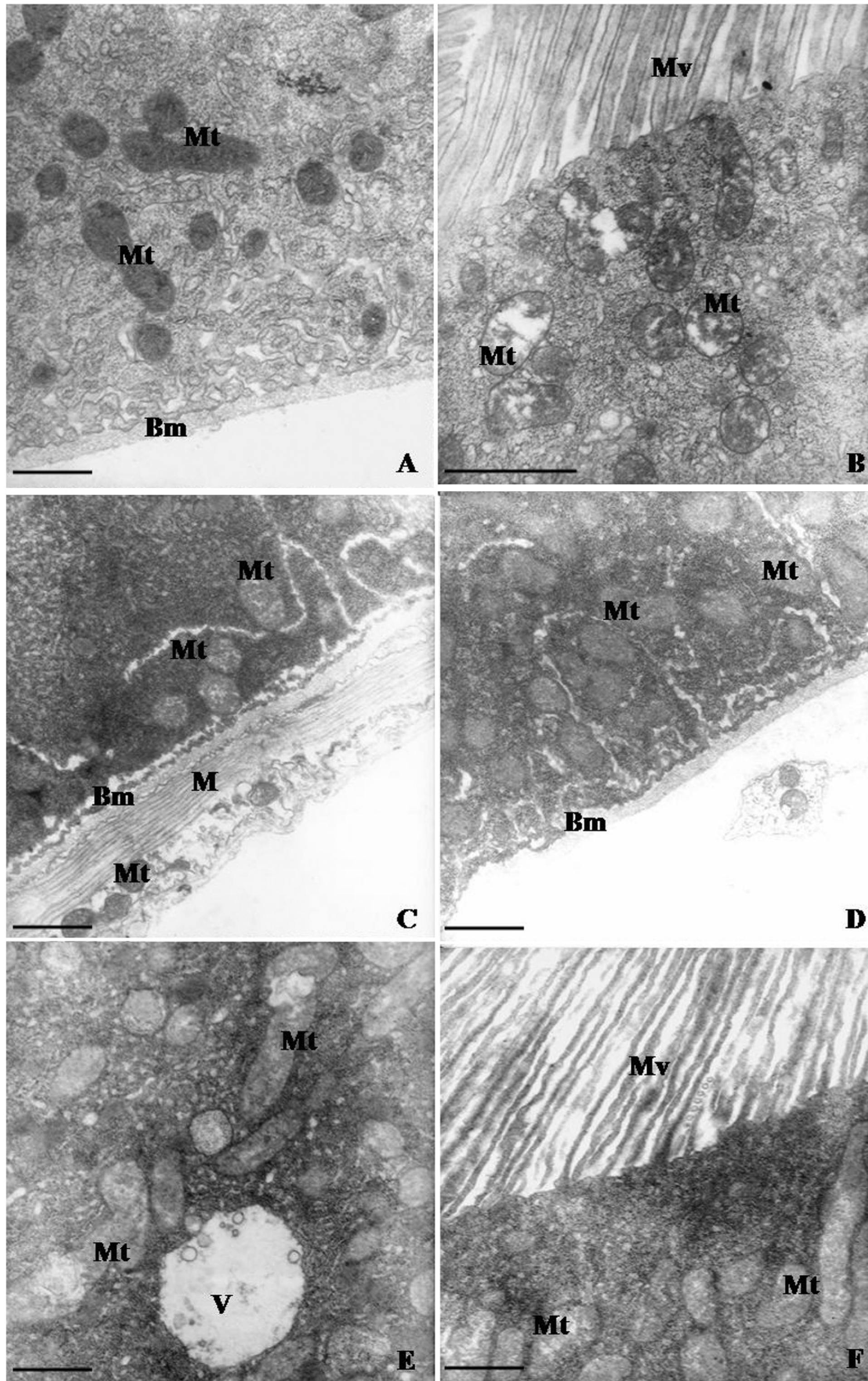

FIG. 4. Transverse ultrathin sections of the posterior midgut from fourth-instar *Culex quinquefasciatus* larvae resistant to the Bin toxin (CqRL1/2362) treated with a mixture of Bin/Cry11Aa toxins at a 3:1 (wt/wt) ratio. (A) Cell from a nontreated larva showing mitochondria of normal aspect (Mt). Bm, basal membrane. (B) Cell from 1-h-treated larvae with mitochondrial crista destruction and vacuolation (Mt); microvilli are intact (Mv). (C) Cell from 1-h-treated larvae showing altered cytoplasm and damaged mitochondria (Mt) at the basal region of the cell in contrast with the normal aspect observed in the same organelle present in a subjacent myoid (M) cell. Bm, basal membrane. (D) Cell from 6-h-treated larvae with mitochondrial (Mt) swollen, dense cytoplasm and preserved basal membrane (Bm). (E) Cell from 6-h-treated larvae showing cytoplasmic vacuoles (V) and mitochondrial vacuolation (Mt). (F) Cell from 6-h-treated larvae with intact microvilli (Mv). Mt, mitochondria. Bars, 1 μm.

vacuolation, probably linked to the presence of Cry11Aa, since this effect was not detected in Bin-toxin-treated larvae (10). Other important alterations detected, such as induction of cytoplasmic vacuoles and the dense aspect of the cytoplasm, are similar to effects caused by the Bin toxin and suggest that, in cells lacking Cqm1 receptor, Bin toxin may act by a synergistic mechanism with Cry11Aa, consistent with studies that demonstrated that the association of Bin toxin and Cry11Aa can overcome resistance (22, 29).

The effects of the Cry48Aa/Cry49Aa mixture on Bin-toxin-resistant larvae were similar to those on the Bin-toxin-susceptible (CqSF) larvae and indicated that the action of the mixture does not rely on the presence of the Cqm1 receptor on the midgut cells. This is consistent with the lack of cross-resistance between these toxins, as indicated by studies with strain IAB59 that were performed prior to the characterization of the Cry48Aa/Cry49Aa toxin (19, 21, 26). Larvae resistant to *B. sphaericus* IAB59 (CqRL2/IAB59) showed minor alterations that were not associated with larval death, similar to results observed in CqRL1/2362 larvae that were treated with Bin toxin (10). The mechanism that underlies Cry48Aa/Cry49Aa resistance in the CqRL2/IAB59 colony is still unknown, although larval survival and lack of relevant cytopathological effects after treatment might indicate that the action of Cry48Aa/Cry49Aa toxin on cells of CqSF and CqRL1/2362 larvae was specific and probably, therefore, receptor mediated. Nevertheless, there may be other mechanisms involved in resistance, such as, for instance, failure in protoxin processing.

Which component carries the receptor binding site or has pore-forming activity is an aspect that remains unknown (13, 14). Cry48Aa, which is a member of the three-domain family of Cry toxins, has, hypothetically, a domain II for receptor binding, in addition to a domain I, which may be involved with pore formation. In turn, Cry49Aa shares similarities with both the receptor binding (BinB) and the pore-forming (BinA) components of the Bin toxin (13). Our data imply an interaction between the Cry48Aa and Cry49Aa components that results in a complex and specific combination of cytopathological effects resulting from the joint action of Bin and three-domain-protein-like insecticidal proteins. It has been demonstrated that resistance selection is inversely proportional to the number of toxins used, particularly those that interact with distinct receptors on midgut epithelial cells (11, 12, 28). The ability of Cry48Aa/Cry49Aa to overcome resistance makes this toxin a promising weapon in the armory of safe, insecticidal proteins with great potential for use as active ingredients of a new generation of biolarvicides for vector control that conjugate toxic factors with distinct sites of action.

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