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Identification of Cry48Aa/Cry49Aa toxin ligands in the midgut of *Culex quinquefasciatus* larvae

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ABSTRACT

A binary mosquitocidal toxin composed of a three-domain Cry-like toxin (Cry48Aa) and a binary-like toxin (Cry49Aa) was identified in *Lysinibacillus sphaericus*. Cry48Aa/Cry49Aa has action on *Culex quinquefasciatus* larvae, in particular, to those that are resistant to the Bin Binary toxin, which is the major insecticidal factor from *L. sphaericus*-based biolarvicides, indicating that Cry48Aa/Cry49Aa interacts with distinct target sites in the midgut and can overcome Bin toxin resistance. This study aimed to identify Cry48Aa/Cry49Aa ligands in *C. quinquefasciatus* midgut through binding assays and mass spectrometry. Several proteins, mostly from 50 to 120 kDa, bound to the Cry48Aa/Cry49Aa toxin were revealed by toxin overlay and pull-down assays. These proteins were identified against the *C. quinquefasciatus* genome and after analysis a set of 49 proteins were selected which includes midgut bound proteins such as aminopeptidases, amylases, alkaline phosphatases in addition to molecules from other classes that can be potentially involved in this toxin's mode of action. Among these, some proteins are orthologs of Cry receptors previously identified in mosquito larvae, as candidate receptors for Cry48Aa/Cry49Aa toxin. Further investigation is needed to evaluate the specificity of their interactions and their possible role as receptors.

1. Introduction

Lysinibacillus sphaericus is an entomopathogen bacterium that can produce crystals containing protoxins with high and selective activity against mosquito larvae, in particular those from the *Culex pipiens* complex. Some insecticidal proteins have been identified in *L. sphaericus* strains (Allievi et al., 2014; Berry, 2012) and the Binary crystal protoxin (Bin), which was the first mosquitocidal factor characterized, remains the active principle of the commercial larvicides based on this bacterium (Silva-Filha et al., 2014). Bin is a heterodimer composed of BinA (42 kDa) and BinB (51 kDa) polypeptides which are produced at high levels in equimolar concentrations by some strains (Charles et al., 1996). Bin achieves the optimal activity only when both components are present, which characterizes its binary nature (Nicolas et al., 1993). Its mode of action has been mostly studied in species from the *Culex pipiens* complex and, after ingestion and proteolytic processing of protoxins, the active BinB subunit is responsible for specifically binding to the Cpm1/Cqm1 α-glucosidases that act as midgut receptors (Darboux et al., 2001; Romão et al., 2006; Silva-Filha et al., 1999), while the BinA component is associated with cell toxicity (Nicolas et al., 1993). Resistance of *C. pipiens* and *C. quinquefasciatus* larvae to Bin toxin has been recorded (Mulla et al., 2003; Nielsen-Leroux et al., 2002; Rao et al., 1995; Wirth et al., 2000; Yuan et al., 2000) due to mutations in genes encoding the receptors, which lead to the production of truncated or non-functional proteins and cause the failure of Bin toxin binding on the midgut epithelium. This has been the major resistance mechanism documented and *cpm1*/*cqm1* alleles causing such failures have been reported (Chalegre et al., 2012, 2015; Darboux et al., 2002, 2007; Darboux et al., 2002; Guo et al., 2013; Romão et al., 2006).

Resistance to *L. sphaericus* based on loss of Bin toxin binding

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highlights the need to characterize molecules with distinct modes of action. Investigation of *L. sphaericus* strain IAB59 began soon after the first reports of Bin-based resistance since this strain is toxic to Bin-resistant larvae, suggesting the production of a novel insecticidal factor that can overcome resistance to Bin toxin (Nielsen-LeRoux et al., 2001; Pei et al., 2002; Yuan et al., 2003). Jones et al. (2007) identified the insecticidal factor as Cry48Aa (135 kDa) and Cry49Aa (53 kDa), which are also produced as protoxins in small crystalline inclusions during sporulation. Cry48Aa/Cry49Aa is considered a new binary toxin produced by *L. sphaericus* since neither the Cry48Aa nor Cry49Aa component shows toxicity to larvae alone (Jones et al., 2007). They can act in synergy forming the complex Cry48Aa/Cry49Aa through the N-terminal portion of the Cry49Aa subunit (Guo et al., 2016). These toxins have comparable toxicity to Bin against *C. quinquefasciatus*, and remain active to Bin-resistant larvae, when both Cry toxins are produced as recombinant proteins and are administered in equimolar ratios (Jones et al., 2008). Unfortunately, native strains are deficient in expression of Cry48Aa and do not attain the optimal 1:1 Cry48Aa:Cry49Aa ratio required for high toxicity, which accounts for why strains such as IAB59 can produce both binary toxins, but are not more toxic than strains producing only Bin toxin.

The Cry48Aa component of the toxin belongs to the three-domain (3D) structural family of Cry proteins with 33% amino acid identity with the Cry4Aa toxin from *Bacillus thuringiensis* svar. *israelensis* (Bti) (Boonserm et al., 2006; Jones et al., 2007). Cry49Aa is part of the group Bin-toxin-like proteins (Toxin−10 family) with about 30% identity to both subunits of the Bin toxin from *L. sphaericus*, in addition to comparable identity to Cry36 (34%) and Cry35 (20%) produced by *B. thuringiensis* strains (Berry, 2012; Jones et al., 2007). The initial steps of the mode of action of Cry48Aa and Cry49Aa are similar to that of the Bin toxin comprising ingestion of crystals, solubilization under alkaline pH and proteolytic activation of protoxins into toxins (Jones et al., 2008), interaction with midgut (de Melo et al., 2009; Guo et al., 2016) followed by cytopathological effects which appear similar to those produced by a synergistic mixture of Cry-like and Bin-like toxins (de Melo et al., 2009). However, the identity of ligands and receptors in the larval midgut that underlie toxic action and larval mortality is still unknown. The investigation of this specific step of the mode of action is strategic since Cry48Aa/Cry49Aa is toxic to Bin-resistant *C. quinquefasciatus* lacking the midgut receptors (Cqm1) for Bin toxin (de Melo et al., 2009; Pei et al., 2002) which indicates that the Cry48Aa/Cry49Aa toxin complex interacts with distinct molecules mediating toxicity to larvae. Recently it was shown that both subunits display the ability to bind to the *C. quinquefasciatus* larval midgut (Guo et al., 2016). In this context, the major goal of the present study was to identify potential ligands for Cry48Aa/Cry49Aa in the midgut of *C. quinquefasciatus* larvae and contribute to the understanding of the mode of action of this mosquitocidal toxin.

2. Materials and methods

2.1. Preparation of cry toxins

Cry48Aa and Cry49Aa were produced individually in the acrystalliferous *Bacillus thuringiensis* svar. *israelensis* strain 4Q7 transformed with plasmids pSTAB135 and pHTP49, which carry genes encoding the respective toxins (Jones et al., 2007). Cultures were grown in sporulation medium (de Barjac and Lecadet, 1976) supplemented with 1% glucose and erythromycin (25 μg/ml), under agitation (200 rpm) at 30 °C, for 72 h until reaching sporulation (≥80%). Spore-crystal biomass was centrifuged, sequentially washed with 1M NaCl/10 mM EDTA pH 8.0 and 10 mM EDTA pH 8.0, and stored at −80 °C. Crystal/spores from both recombinant Bt strains were also processed together. For this purpose biomass containing spore-crystals of each protein were combined (1:1 wt/wt), solubilized (50 mM NaOH, 30 °C, 1 h, at 150 rpm), the supernatant containing both solubilized proteins was separated by centrifugation (21.000*g*, 4 °C, 30 min) and the pH was adjusted to ≈8.5 using 0.1 M HCl. Combined protoxins were activated with pancreatic bovine trypsin (1:100 wt/wt, 30 °C, 1 h). Supernatant, containing the activated proteins, was centrifuged as described above and then dialyzed (0.02 M sodium phosphate, pH 8, 4 °C, 16 h). Protein concentration was determined according to Bradford (1976) using the Biorad reagent (Biorad, Hercules, CA, USA) and a bovine serum albumin standard curve. Size and the integrity of proteins were analyzed in 10% SDS-PAGE. Activated proteins were stored at −80 °C. Moreover, in this study Cry49Aa protein fused to a C-terminal poly-histidine tag (Cry49Aa-His) was individually produced in *Escherichia coli* T7 express cells (New England Biolabs, Beverly, MA, USA). For this purpose, the *cry49Aa* gene was amplified from pHTP49 (described above) using specific primers containing *Bam*HI (bold) and *Not*I (underlined) restriction sites (Fwd 5′-CGA**GGATCC**ATGGAAAATCAAATAAAAGAAGAATTTAAC-3′, Rev 5′-CGAGCGGCCGCATTATAATATGGCTTTGAATTTTCATG-3′) and subsequently cloned into the expression vector pET21a® (Novagen, USA). Antibodies against the His-tag were used to track binding of the Cry48Aa/Cry49Aa mix through the Cry49-His. Attempts to produce Cry48Aa in *E. coli* in order to have a suitable expression to evaluate this toxin were not successful. Cry49Aa cultures of transformed T7 express cells were grown using Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) (under agitation, 200 rpm, 37 °C until reaching an OD_{600} of 0.5), and induced with IPTG (0.1 mM, 30 °C, 4 h). Cultures were centrifuged (21.500*g*, 4 °C, 10 min), the cell pellets were re-suspended in phosphate-buffered saline pH 7.4 (PBS), sonicated, and Triton X-100 was added to a final 1% vol/vol. The samples were centrifuged (1700*g*, 30 min, 4 °C) and proteins from the supernatants were purified using the Ni-NTA resin (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Proteins were eluted with 1M imidazole in wash buffer (50 mM sodium phosphate-buffer, 300 mM NaCl, 10% glycerol, pH 6, 4 °C, 1 h) and dialyzed (50 mM dibasic sodium phosphate, 5 mM monobasic sodium phosphate, 50 mM NaCl, 0.1% Triton x-100, 5% glycerol, pH 7.8, 4 °C, overnight). Protein integrity and concentration were verified, as previously described. The purified Cry49Aa-His from *E. coli* was combined with solubilized Cry48Aa from Bt in 1:2 ratio (μg protein: μg protein) and the mixed sample was subjected to *in vitro* processing, as previously described. The concentration and integrity of the activated mix of proteins was verified by 10% SDS-PAGE and then it was stored at −80 °C. Cry49Aa fused to glutathione S-transferase (Cry49Aa-GST) was also individually produced in *E. coli* BL21 Star™ (DE3) cells (ThermoFischer Scientific, Waltham, MA, USA). *cry49Aa* gene was amplified from pHTP49 using the following primers containing *Bam*HI and *Not*I sites as described (Fwd 5′-CGA**GGATCC**ATGGAAAATCAAATAAAAGAAGAATTTAAC-3′, Rev 5′- CGAGCGGCCGCTTAATTATAATATGGCTTTGAATTTTCATG -3′). The BinB subunit of the binary toxin fused to glutathione S-transferase (BinB-GST) was also produced according to Romão et al. (2006). Further steps to produce purified GST proteins are also described in that study.

2.2. Mosquito strain

Fourth instar larvae of the CqSLab *Culex quinquefasciatus* strain were used in this study. This colony has been maintained in the