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Single nucleotide deletion of *cqm1* gene results in the development of resistance to *Bacillus sphaericus* in *Culex quinquefasciatus*

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ABSTRACT

The entomopathogen *Bacillus sphaericus* is one of the most effective biolarvicides used to control the *Culex* species of mosquito. The appearance of resistance in mosquitoes to this bacterium, however, remains a threat to its continuous use in integrated mosquito control programs. Previous work showed that the resistance to *B. sphaericus* in *Culex* colonies was associated with the absence of the 60-kDa binary toxin receptor (Cpm1/Cqm1), an alpha-glucosidase present in the larval midgut microvilli. In this work, we studied the molecular basis of the resistance developed by *Culex quinquefasciatus* to *B. sphaericus* C3-41. The *cqm1* genes were cloned from susceptible (CqSL) and resistant (CqRL/C3-41) colonies, respectively. The sequence of the cDNA and genomic DNA derived from CqRL/C3-41 colony differed from that of CqSL one by a one-nucleotide deletion which resulted in a premature stop codon, leading to production of a truncated protein. Recombinant Cqm1S from the CqSL colony expressed in *Escherichia coli* specifically bound to the Bin toxin and had α-glucosidase activity, whereas the Cqm1R from the CqRL/C3-41 colony, with a deletion of three quarters of the receptor's C-terminal lost its α-glucosidase activity and could not bind to the binary toxin. Immunoblotting experiments showed that Cqm1 was undetectable in CqRL/C3-41 larvae, although the gene was correctly transcribed. Thus, the *cqm1R* represents a new allele in *C. quinquefasciatus* that confers resistance to *B. sphaericus*.

1. Introduction

Mosquitoes transmit serious human diseases, such as malaria, yellow fever, West Nile fever and dengue. Targeting the mosquitoes hosts is recognized as an effective approach for eradication of the threat from these tropical diseases. Due to the high larvicidal activities of insecticidal binary (Bin) protoxin produced as crystalline inclusions during bacterial sporulation (Federici et al., 2007; Lacey, 2007; Nicolas et al., 1993; Park et al., 2009), highly toxic *Bacillus sphaericus* strains have been effectively used for the control of the genera *Culex* and *Anopheles* (Merritt et al., 2005). The Bin toxin, is composed of two subunits named BinA (42 kDa) and BinB (51 kDa) (Arapinis et al., 1988; Berry and Hindley, 1987), which act in synergy and present optimal toxicity at equimolar concentration (Nicolas et al., 1993). The mode of action of the Bin toxin depends on toxins solubilized in the alkaline pH of the larval midgut, which are released, followed by activation of both BinA and BinB proteins by larval proteases into toxic fragments of 39 and 43 kDa, respectively (Broadwell and Baumann, 1987). The activated toxin, through its BinB subunit, specifically binds to a single class of specific receptors, and the BinA subunit confers toxicity, which triggers a set of cytopathological events resulting in larval death (Charles, 1987; de Melo et al., 2008; Singh and Gill, 1988). Three kinds of receptor such as Cpm1 (*Culex pipiens*) (Darboux et al., 2001), Cqm1 (*Culex quinquefasciatus*) (Romão et al., 2006) and Agm3 (*An. gambie*) (Opota et al., 2008) have been sequenced and characterized in susceptible mosquito species. All of them are 60-kDa α-glucosidases located on the apical membrane of midgut epithelium cells by a glycosylphosphatidylinositol (GPI) and have high binding affinity to the Bin toxin. It is likely that the toxin is able to form pores on cell membrane, although the mechanisms of larval death are still unknown.

Insecticides based on *B. sphaericus* were developed for mosquito control in breeding sites, where its residual activity is important. However, *B. sphaericus* is at risk for inducing resistance in mosquitoes because it targets a single receptor type in the larval midgut. Attempts to select *Culex* colonies under strong selection pressure with *B. sphaericus* strains 2362 and C3-41 demonstrated the potential for development of resistance, under laboratory conditions (Pei et al., 2002; Wirth et al., 2000a,b). The occurrence of resistance among field *Culex* populations submitted to intensive *B. sphaericus* treatment had also been recorded (Nielsen-Leroux et al., 1995, 2002; Rao et al., 1995; Yuan et al., 2000). Previous studies

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demonstrate that the absence and alteration of the functional receptors in the midgut leading to the failure of the Bin toxin to bind to the receptors are the major cause of mosquito larvae achieving high level of resistance to *B. sphaericus* (Darboux et al., 2002; Nielsen-Leroux et al., 1995; Oliveira et al., 2004; Romão et al., 2006). So far, six *cpm1/cqm1* resistance alleles of different origins have been found in *Culex* populations. The $cpm1_{GEO}$ allele (California), is due to a nonsense mutation resulting in a truncated protein, eliminating the GPI anchorage of the receptor to the membrane (Darboux et al., 2002). The $cpm1_{BP}$ and $cpm1_{BP}$-*del* alleles were found in the *C. pipiens* BP population from France (Chevillon et al., 2001), one had a single nucleotide mutation preventing the expression of the Bin receptor causing the lack of toxin binding on the epithelial cell membrane, while the other involved a transposon-mediated mutation leading to the creation of a new intron encoding a truncated protein which cannot interact with the Bin toxin (Darboux et al., 2007). In *C. quinquefasciatus* originating from the city of Recife (Brazil), three resistance alleles (corresponding to 16-, 19- and 25-nucleotides (nt) deletions) were found in *cqm1*. Among them, $cqm1_{REC}$ was associated with the resistance in the laboratory selected populations, and $cqm1_{REC\text{-}D16}$ and $cqm1_{REC\text{-}D25}$ were detected in *B. sphaericus* nontreated and treated field populations, respectively. The three alleles introduced equivalent frameshifts in the sequence and generated premature stop codons, and resulted in the production of truncated proteins unable to localize on the midgut (Chalegre et al., 2012; Romão et al., 2006). The elucidation of mutations conferring resistance to the Bin toxin is essential for the assessment of larval genotype for *cqm1* and $cqm1_{REC}$ alleles in larvae. Furthermore, the other resistance mechanism is entirely not related to the toxin-receptor-binding step. This unknown mechanism might play a determinant role in toxicity and were not elucidated to date. (Nielsen-Leroux et al., 1997).

B. sphaericus C3-41, belonging to flagellum serotype H5a5b like strains 2362 and 1593 (Yuan et al., 1999), is a highly active strain that was isolated from a mosquito-breeding site in China in 1987. In certain southern cities in China, this formulation has been intensively used as the sole larvicide to treat mosquito breeding sites for more than 20 years. Under laboratory conditions, after 18 generations of exposure to high concentrations of *B. sphaericus* C3-41, a susceptible colony developed high level resistance (resistance ratio [RR], >140,000-fold) (Pei et al., 2002). For the management of mosquito resistance, it is essential to understand the full range of potential resistance mechanisms. The major goal of this work was to investigate the molecular basis for CqRL/C3-41 colony conferred high level resistance to *B. sphaericus* C3-41. In this work, we showed that a single nucleotide deletion mutation (generating a premature stop codon) in the *cqm1R* sequence from resistance colony resulted in the production of a truncated form of the receptor and provoked the absence of Cqm1 on midgut microvilli. These results provide an important information for further understanding the mechanism of resistance in mosquitoes and the resistance management.

2. Materials and methods

2.1. Mosquito colonies

A susceptible laboratory *C. quinquefasciatus* colony (CqSL), which was established from a laboratory-reared colony more than 10 years ago, was reared without exposure to *B. sphaericus* and did not show any marked change in its susceptibility to *B. sphaericus* C3-41. A resistant laboratory *C. quinquefasciatus* colony (CqRL/C3-41) was derived from the CqSL colony and selected with *B. sphaericus* strain C3-41 under laboratory conditions, with a resistance ratio of more than 140,000-fold (Pei et al., 2002). Larvae of all colonies were reared in enamel pans filled with dechlorinated tap water and fed with a mixture of yeast powder and wheat mill or cat chow. The pupae were removed from the pans every day and placed in screen cages for emergence. The adults were allowed to feed on 10% sucrose solution, and the females were fed with blood from mice. All larvae and adults were held at 26–28 °C and a photoperiod of 12:12 h (light–dark).

2.2. Preparation of brush border membrane fractions

The brush border membrane fractions (BBMF) were obtained from 4th instar larvae frozen and stored without buffer, at −71 °C. BBMF were prepared by the differential magnesium precipitation method (Silva-Filha et al., 1997), resuspended in ice-cold buffer A (0.3 M mannitol, 5 mM EGTA, 20 mM Tris–Cl, pH 7.5), and the concentration of total protein was measured with a bicinchoninic acid (BCA) kit (Thermo). The activities of BBMF, α-glucosidase (EC3.2.1.20) and leucine aminopeptidase (EC3.4.11.1) were determined as previously described (Silva-Filha et al., 1999). Freshly prepared BBMF were kept on ice and used the same day.

2.3. Expression and purification of putative Bin toxin receptor

Susceptible and resistant *C. quinquefasciatus* RNA extraction were carried out from pools of 4th instar larvae using Trizol reagent, as previously described (Romão et al., 2006). The genomic DNA of 4th instar larvae was extracted and purified as previously described (Ayres et al., 2002). Reverse transcription was performed at 37 °C, for 1 h using 10 μg of total RNA, 10 U of M-MLV reverse transcriptase (Invitrogen) and 2 mM of the Oligo dT18 primer (Invitrogen). Specific oligonucleotides primers were designed based on the previously published *cqm1* cDNA sequence (Romão et al., 2006). PCR reactions were performed to amplify the *cqm1* cDNA and genomic DNA using specific primers Fwd (5′ ATTAGAATTCATGCGACCGCTGGGAGCTTTG 3′, *Eco*RI site is underlined), and Rev (5′ GCCCAAGCTTTTACACGAAAATATACCTGGC 3′, *Hin*dIII site is underlined). The genes encoding the *cqm1S* (from CqSL colony) and *cqm1R* (from CqRL/C3-41 colony) were introduced into pET-28a (Novagen), a His tag expression vector, resulting in recombinant plasmids pET-cqm1S and pET-cqm1R. The cloned genes were sequenced, and the nucleotide and amino acid sequences were analyzed by using BLAST and CLUSTALW.

Plasmids pET-cqm1S and pET-cqm1R were transformed into *Escherichia coli* BL21 competent cells. Bacterial clones harboring the different plasmids were grown at 37 °C in Luria–Bertani medium containing kanamycin (50 μg/ml) until the optical density at 600 nM (OD_{600}) of the culture reached 0.3–0.5. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM for 4 h. Cells were harvested by centrifugation (10,000 g, 4 °C) and recombinant proteins were purified by using a His-Bind Resin chromatography kit (Novagen) following the manufacturer's procedure. The collected eluted solution was dialyzed in PBS at 4 °C. Protein concentrations were determined by using a BCA kit (Thermo). The activity of α-glucosidase was determined as previously described (Darboux et al., 2001).

2.4. Expression and purification of BinA and BinB proteins

The genes encoding both the BinB active core subunit and BinA subunit were amplified from *B. sphaericus* C3-41 using specific primers binBc-Fwd (5′ CGGGATCCCTTCCAGAAATATCAAAA 3′, *Bam*HI site is underlined), and binBc-Rev (5′ CGCTCGAGTTTTGTTAGCGTAAGTG 3′, *Xho*I site is underlined), as well as binA-Fwd (5′ CGGGATCCATGAGAAATTTGGATTTTATTG 3′, *Bam*HI site is underlined) and binA-Rev (5′ CGCTCGAGTTAGTTTTGATCATCTGTAAT 3′, *Xho*I site is underlined). The amplified genes were