Sussex Research Online

Identification of Aedes aegypti specificity motifs in the N-terminus of the Bacillus thuringiensis Cry2Aa pesticidal protein

Article (Published Version)

Goje, Lazarus Joseph, Elmi, Elmi Dahir, Bracuti, Anthony, Courty, Thomas, Rao, Tejas, Alzahrani, Faisal Ay and Crickmore, Neil (2020) Identification of Aedes aegypti specificity motifs in the N-terminus of the Bacillus thuringiensis Cry2Aa pesticidal protein. Journal of Invertebrate Pathology, 174. a107423 1-5. ISSN 0022-2011

This version is available from Sussex Research Online: http://sro.sussex.ac.uk/id/eprint/91951/

This document is made available in accordance with publisher policies and may differ from the published version or from the version of record. If you wish to cite this item you are advised to consult the publisher's version. Please see the URL above for details on accessing the published version.

Copyright and reuse:
Sussex Research Online is a digital repository of the research output of the University.

Copyright and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable, the material made available in SRO has been checked for eligibility before being made available.

Copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational, or not-for-profit purposes without prior permission or charge, provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

http://sro.sussex.ac.uk

Identification of *Aedes aegypti* specificity motifs in the N-terminus of the *Bacillus thuringiensis* Cry2Aa pesticidal protein.

Lazarus Joseph Goje, Elmi Dahir Elmi, Anthony Bracuti, Thomas Courty, Tejas Rao, Faisal Ay Alzahrani, Neil Crickmore*

School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK

*corresponding author n.crickmore@sussex.ac.uk

Abstract

One advantage of using the Cry proteins of *Bacillus thuringiensis* as pesticides is their relatively narrow spectrum of activity, thus reducing the risk of non-target effects. Understanding the molecular basis of specificity has the potential to help us design improved products against emerging pests, or against pests that have developed resistance to other Cry proteins. Many previous studies have associated specificity with the binding of the Cry protein, particularly through the apical regions of domain II, to particular receptors on the midgut epithelial cells of the host insect. We have previously found that the specificity of Cry2A proteins against some insects is associated with domain I, which is traditionally associated with pore-formation but not receptor binding. In this work we identify four amino acids in the N-terminal region that, when mutated, can confer activity towards *Aedes aegypti* to Cry2Ab, a protein known to lack this toxicity. Intriguingly these amino acids are located in the region (amino acids 1-49) that is believed to be removed during proteolytic activation of the Cry protein. We discuss how the motifs containing these amino acids might be involved in the toxic process.

Keywords

Cry toxin, Yellow fever mosquito, proteolytic activation

1 Introduction

The Cry pesticidal proteins from *Bacillus thuringiensis* have been widely adopted as insecticides in the agricultural sector, either as sprayable products or incorporated into genetically modified crops. Individual Cry proteins tend to have a relatively narrow spectrum of activity (van Frankenhuyzen, 2009) although some have broader ranges spanning multiple orders of insects (van Frankenhuyzen, 2013). The Cry2A proteins are examples of those with activity spanning multiple orders, for example Cry2Aa has long been known to have activity against both lepidopteran and dipteran insects (Donovan et al., 1988). Although Cry2Ab is also known to have activity against both lepidopteran and dipteran species it is well established that unlike Cry2Aa it has no activity against the mosquito *Aedes aegypti* (Donovan et al., 1988; McNeil and Dean, 2011). Previous studies had made use of this observation to identify regions within the Cry2A toxins that were responsible for the specificity towards each type of insect. The creation of hybrids between Cry2Aa and Cry2Ab identified a region within domain II (amino acids 307-382) that was believed to be responsible for conferring activity to *A. aegypti* (Widner and Whiteley, 1990). Later work (Liang and Dean, 1994) made use of the differential toxicity of Cry2Aa and Cry2Ab towards the lepidopteran insect *L. dispar* to define regions responsible for specificity to both this insect (amino acids 340-412) and to *A. aegypti* (amino acids 278-412). When the structure of Cry2Aa was solved it was observed that these specificity regions could form a putative receptor binding epitope, a structure that in the protoxin is obscured by the N-terminus of protein but potentially revealed upon protoxin activation (Morse et al., 2001). A consensus was therefore forming that as with other three-domain Cry proteins the specificity of the

43 Cry2 proteins was determined by a receptor binding motif within domain II. More recent work
44 however has implicated domain I of Cry2A in specificity determination based on the observation that
45 in several natural and artificial hybrids, activity was associated with domain I and not domain II (Shu
46 et al., 2017). The specificity of a Cry protein towards a particular insect can be determined by
47 multiple factors (Jurat-Fuentes and Crickmore, 2017) although receptor binding is still believed to be
48 the dominant one. The use of directed evolution has shown that it is possible to modify a toxin to
49 bind to a new receptor and thus alter its toxicity spectrum (Badran et al., 2016). This study aimed to
50 better understand the nature of specificity, an understanding which could facilitate the future design
51 of bespoke pesticidal proteins.

52 **2 Materials and methods**

53 *2.1 Strains and plasmids*

54 The *E. coli* strain DH5α was used for general manipulation procedures while the strain BL21(DE3)
55 pLysS was used for protein expression. *Aedes aegypti* were cultured in house and the adults fed on
56 20% sucrose or defibrinated horse blood and maintained under a 18:6 hour photoperiod at 23°C.
57 The genes encoding the Cry2A proteins were cloned into the pGEM T-easy vector (Promega) under
58 the control of the T7 promoter. Plasmids for the expression of Cry2Aa and Cry2Ab in *B. thuringiensis*
59 have been described previously (Crickmore and Ellar, 1992; Crickmore et al., 1994).

60 *2.2 Construction of the hybrids and mutants*

61 Hybrids were created by blunt-end ligation of PCR amplified specific domains, using 5'
62 phosphorylated primers, with plasmids containing a *cry2A* gene lacking that domain which were
63 produced by inverse PCR. PfuUltra II hotstart PCR master mix (Agilent) was used for all amplification
64 reactions and followed the manufacturer's recommended protocols. Prior to purification of
65 amplification products by a Qiaquick kit (Qiagen), template DNA was destroyed by digestion with
66 DpnI. Mutations were created by inverse PCR using 5' phosphorylated primers. All primers used in
67 this study are listed in Supplementary table 1.

68 *2.3 Bioassay*

69 The standard bioassay method defined by the World Health Organization (WHO, 2005) was used in
70 this study. This involved using 4 ml of deionised water per larvae. To 80 ml of deionised water (with
71 or without Cry2 protein) in a 100 ml beaker, 20 late 3rd instar larvae were carefully added using a
72 pipette dropper. They were then maintained for a period of 24 to 72 hours at 27^0C with an 18 light;
73 6-hour dark photoperiod. A small amount of larval food was added to each beaker. The number of
74 dead larvae in each beaker was counted after periods of 24, 48 and 72 hours. During the counting of
75 larvae, moribund larvae that were incapable of rising to the surface of the water, or did not show
76 the characteristic diving reaction when the water was disturbed, were counted as dead. The result
77 was expressed as percentage mortality. If more than 10% of the control larvae pupated or if more
78 than 20% mortality was recorded in the control, the test was discarded and repeated. In the
79 calculation of percentage mortality the percentage of dead larvae in the control was factored in to
80 the values from the treatment groups.

81 *2.4 Preparation of Aedes aegypti midgut extract*

82 Proteases from *Aedes* midguts were prepared by extracting 50 midguts from late third instar larvae
83 through dissection. The motility of the larvae was restricted by placing them on ice for five minutes
84 prior to dissection. The 50 dissected guts were suspended in 100 µl of 1X PBS buffer and
85 homogenized by sonication. The resulting mixture contained both extracellular and intracellular

86 fluids due to the difficulty of extracting just the gut contents from the small guts. The homogenized
87 material was centrifuged for 10 minutes at 6268 x g at 4^0C. The supernatant was decanted to a 1.5
88 ml Eppendorf tube and stored at 4^0C for future use while the pellet was discarded.

89 *2.4 Protein expression, solubilisation and proteolytic cleavage*

90 For the *E. coli* transformants cells were grown in 2xLB broth at 37°C until an OD600 of 0.4-0.6 was
91 reached at which point IPTG was added to a concentration of 0.5mM and incubation continued
92 overnight at 25°C. Crystals were harvested by centrifugation, then sonication in water, before a final
93 resuspension in water. *B. thuringiensis* transformants were grown on LB plates for 72 hours at 30°C
94 until sporulated and then the spores and crystals were stored in water following sonication. Cry2A
95 crystals were solubilized in 50 mM sodium hydroxide at 37°C for 1 hour and the solubilized protein
96 recovered from the supernatant following centrifugation. Chymotrypsin or *A. aegypti* gut extract
97 (both in PBS) was mixed with the solubilized protein at a 1:1 v/v ratio and incubated at 37°C for 1
98 hour.

99 *2.5 Ni-NTA binding assays*

100 400µl of Ni-NTA bead slurry in a gravity flow column was washed five times with equilibration
101 buffer (50mM NaOH, 1X PBS pH 9). 100µl of protein sample was then applied to the equilibrated
102 column which was then capped and incubated for 1 hour at 4°C with frequent shaking. Flow
103 through was collected then the column was washed five times with 200 µl of wash buffer
104 (50mM NaOH, 1X PBS, 2mM imidazole pH9). 50 µl of elution buffer (50mM NaOH, 1X PBS,
105 150mM imidazole pH. 9) was applied to the column and left for 5 minutes before fractions were
106 collected.
107

108 **3 Results**

109 *3.1 Association of domain I with Aedes aegypti activity.*

110 Since our previous work (Shu et al., 2017) had indicated that Domain I of the Cry2A proteins could
111 influence the specificity of these toxins towards particular insects, including *A. aegypti*, we tested a
112 wider range of proteins against this insect in an attempt to associate activity with particular
113 structural motifs. The nine toxins listed in table 1 were tested at a discriminatory dose of 2 mg/L,
114 those showing less than 10% mortality were considered non-toxic. As previously observed (Shu et
115 al., 2017) Cry2Aa was toxic but the variant Cry2Aa17 with a domain I resembling that of Cry2Ab had
116 no activity. Cry2Ac and Cry2Am were toxic and all the others non-toxic (table 1). Having previously
117 shown that the specificity of Cry2Aa to *A. aegypti* could be transferred to Cry2Ab through the
118 exchange of domain I we wanted to establish whether the same was true for Cry2Ac. Two hybrids
119 were created through the exchange of the first 263 amino acids and the resulting hybrids were
120 tested against *A. aegypti* at a dose of 2mg/L. The hybrid Cry2 AcAbAb containing domain I of Cry2Ac
121 and domains II & III of Cry2Ab gave a mortality of 30% whereas the reciprocal hybrid Cry2 AbAcAc
122 was non-toxic (table 1). These results were consistent with the previous finding that domain I
123 influences activity against this mosquito.

124 *3.2 Aedes aegypti activity associates with the N-terminal 49 amino acids*.

125 Many previous studies on the mechanism of action of Cry toxins had proposed that the specificity of
126 these toxins was largely determined through the interaction of exposed loops in domain II with
127 receptors on the surface of midgut epithelial cells (Jurat-Fuentes and Crickmore, 2017). The
128 structure of Cry2Aa (Morse et al., 2001) reveals that the N-terminal region folds back onto domain II
129 and so we hypothesized that the very N-terminal region of Cry2Aa was functionally part of domain II

130 and participated in receptor binding. To test this the N-terminal region that folds back onto domain
131 II (amino acids 1-49) was exchanged between Cry2Aa and Cry2Ab. The results showed that
132 transferring just the first 49 amino acids from Cry2Aa was enough to confer *A. aegypti* activity to
133 Cry2Ab (table 1). When however, this N-terminal region of Cry2Aa was replaced with the equivalent
134 from Cry2Ab activity was lost. The same result was observed (table 1) when Cry2Ac was used instead
135 of Cry2Aa supporting our hypothesis that this region was crucial for *A. aegypti* activity.

136 *3.3 Identification of four amino acids that associate with A. aegypti activity*.

137 To attempt to identify particular amino acids that might associate with the mosquitocidal activity a
138 multiple sequence alignment of the nine native proteins listed in table 1 was performed. Figure 1
139 shows that at four positions there was a potential association with activity. In the three toxic
140 proteins there is a glutamic acid (E) at position 27 whereas the non-toxic proteins all have a
141 glutamine (Q) here and at positions 43-45 the toxic proteins have a triad of RTD whereas in the non-
142 toxic ones KNN or KDN is found. In order to test whether any of these amino acids were important
143 for *A. aegypti* activity further mutants were made. In these mutants the amino acids in the non-toxic
144 Cry2Ab were replaced with the corresponding amino acids from Cry2Aa. Three mutants were initially
145 made changing either Q27 to E, KNN43-45 to RTD or both together. The results (table 1) show that if
146 all four amino acids are substituted then Cry2Ab acquires *A. aegypti* activity. Reciprocal mutants
147 were also made which showed that making any change in Cry2Aa resulted in the loss of activity
148 (table 1).

149 *3.4 All four amino acids are crucial for A. aegypti activity*.

150 The above data show that both the changes at amino acid 27 and the 43-45 triad are necessary for
151 Cry2Ab to acquire activity. To establish whether all three of the triad are required, the amino acids
152 were mutated individually or in pairs (table 1). None of the resulting mutants showed any activity
153 against *A. aegypti* suggesting that all four are necessary for toxicity to this insect. The importance of
154 these amino acids for toxicity was not consistent with previous reports that removing the N-terminal
155 region of Cry2Aa had no significant effect on toxicity. Ohsawa et al observed that Cry2Aa treated
156 with chymotrypsin, which cleaved the protein after amino acid 144, was still active against the
157 lepidopteran insects *B. mori* and *L. dispar* (Ohsawa et al., 2012). Another report found that
158 genetically deleting the first 42 or 70 amino acids of Cry2Aa did not reduce its activity against three
159 other lepidopteran insects (Mandal et al., 2007). As a further test of the importance of the four
160 identified amino acids for toxicity against *A. aegypti* we created a construct in which the first 45
161 amino acids were deleted. The resulting protein had no activity against *A. aegypti* (table 1).

162 *3.5 Effect of chymotrypsin activation on the activity of Cry2Aa against A. aegypti*

163 Since a previous report (Ohsawa et al., 2012) had shown that Cry2Aa retained lepidopteran activity
164 upon cleavage with chymotrypsin we tested whether the same was true for *A. aegypti*. Cry2Aa
165 protein was digested with three concentrations of chymotrypsin and analysed by SDS PAGE (figure
166 2A). At the highest concentration a single band of around 50kDa was observed, at the lower
167 concentrations partial digestion resulted in a band of around 58kDa sizes consistent with previous
168 studies in which Cry2A had been cleaved with chymotrypsin or lepidopteran gut extracts (Audtho et
169 al., 1999; Ohsawa et al., 2012; Xu et al., 2016). Bioassays were undertaken to compare the activity of
170 native Cry2Aa crystals, solubilized Cry2Aa and fully activated Cry2Aa. Crystalline and solubilized
171 forms of the toxin showed good activity (>50% mortality) at a concentration of 2mg/L however the
172 chymotrypsin activated protein was non-toxic at this concentration. This result suggested that the
173 mode of action of Cry2Aa against *A. aegypti* is significantly different to that against at least some

174 other lepidopteran insects in that there appears to be an absolute need for the N-terminal 45 amino
175 acids. Based on the above we hypothesized that in *A. aegypti* the N-terminal region remains intact
176 when the toxin is ingested by the insect and is not cleaved by the digestive enzymes in the same way
177 that various lepidopteran gut extracts have been shown to cleave it (Audtho et al., 1999; Xu et al.,
178 2016). To test this Cry2Aa was digested with various concentrations of chymotrypsin or *A. aegypti*
179 gut extract and analysed by SDS PAGE (figure 2B). In order to get a higher yield of cleaner protein *B.*
180 *thuringiensis* derived Cry2Aa was used from this point onwards. The results with chymotrypsin were
181 the same as seen in figure 2 with partial digestion seen at higher dilutions of the enzyme. With *A.*
182 *aegypti* gut extract the major band seen corresponded to the circa 58kDa chymotrypsin produced
183 band. At higher dilutions this band disappeared, and only undigested protein was observed.
184 Although a weak band can be seen at around 50kDa the intensity of this band does not vary with
185 enzyme concentration and is also present in the solubilized sample so we do not believe this
186 represents a digestion product. From this experiment we concluded that *A. aegypti* gut extract does
187 not digest Cry2Aa down to the 50kDa band, however digestion is seen to the 58kDa band which has
188 previously been reported as representing cleavage after amino acid Y49 (Audtho et al., 1999;
189 Ohsawa et al., 2012). The hypothesis that the N-terminal region was not cleaved in *A. aegypti* was
190 therefore not supported.

191 *3.6 The cleaved N-terminus does not remain attached to the rest of the Cry2A protein*

192 Having established that the N-terminal 45 amino acids are crucial for *A. aegypti* activity yet a
193 fragment containing these is cleaved off by gut enzymes we wanted to test whether this fragment
194 could remain attached following cleavage and therefore still participate in receptor binding.
195 Although a previous report (Morse et al., 2001) had speculated that the N-terminal region has to be
196 removed to reveal a receptor binding motif this mode of action may apply to insects other than *A.*
197 *aegypti*. It has previously been observed that gut extracts of other insects cleave Cry2A to a 50kDa
198 product (Audtho et al., 1999; Ohsawa et al., 2012; Xu et al., 2016) whereas *A. aegypti* extracts only
199 cleave to a 58kDa product. It is possible therefore that the 144 amino acids removed in producing
200 the 50kDa product are released from the protein structure whereas cleavage at just amino acid 49
201 does not result in release. To test this we created an N-terminally His-tagged Cry2Aa protein and
202 introduced an L144A mutation to prevent chymotrypsin producing the 50kDa band. To establish
203 whether or not the entire protein could be pulled down with the tag following cleavage this
204 recombinant protein was digested with chymotrypsin to produce a mixture of undigested and 58kDa
205 proteins (figure 3A lane 1). When this digest was passed though a Ni-NTA column the 58kDa band
206 appeared in the washes whereas the undigested protein was retained on the column and only
207 observed after elution with imidazole (figure 3A). This result suggested that contrary to our
208 speculation the N-terminal fragment did not remain attached to the rest of the protein following
209 cleavage to the 58kDa band. The experiment was repeated using Cry2Aa digested by *A. aegypti* gut
210 extract (figure 3B) and again indicated that the N-terminal fragment does not remain attached.

211 **4 Discussion**

212 The results presented here confirm our previous finding that sequences at the N-terminal part of the
213 Cry2A proteins influence its specificity, and in particular to the yellow fever mosquito *Aedes aegypti*.
214 Furthermore we have shown that changing just four amino acids can broaden the host range of
215 Cry2Ab to include *A. aegypti*. The ability to add mosquito activity to a Cry protein naturally lacking
216 such an activity has been reported before (Abdullah et al., 2003; Liu and Dean, 2006). In these two
217 cases activity against the common house mosquito *Culex pipiens* was introduced through changing
218 the sequences found at the apex loops of domain II. Given also that previous studies had identified
219 sequences within domain II as being responsible for the mosquitocidal activity of Cry2Aa (Liang and

220 Dean, 1994; Widner and Whiteley, 1990) the involvement of domain I motifs was unexpected. Figure
221 4 compares the structure of Cry2Aa with a modelled structure of Cry2Ab with the four amino acids
222 required to give *A. aegypti* activity to Cry2Ab highlighted. As can be seen the four amino acids are all
223 surface exposed and could potentially be directly involved in receptor binding. The RTD triad in
224 particular can easily be envisaged as co-operating with the domain II sequences in binding to a cell
225 surface receptor. The importance of amino acid 27 is less intuitive, it can be seen that a cavity
226 present in Cry2Aa is predicted to be blocked in Cry2Ab so one could speculate that this cavity is
227 important for receptor docking. When the structure of Cry2Ab Q27E KNN43-45RTD was modelled
228 the cavity was observed to be present (data not shown). It should be noted that these four amino
229 acids are not sufficient for activity against *A. aegypti*, others will be crucial for receptor binding and
230 of course for pore-formation. We do suggest though, that this configuration of amino acids is
231 required for significant *A. aegypti* activity. This distinction provides an explanation for the earlier
232 findings that the transfer of sequences from domain II of Cry2Aa could confer some (albeit weak) *A.*
233 *aegypti* activity to Cry2Ab. If motifs present in both domain II (for example the apex loops) and at
234 the N-terminus are required for activity then the presence of some components of these structural
235 motifs could be sufficient for the protein to demonstrate some level of activity.

236 The observation that these *A. aegypti* specificity motifs were located in an N-terminal region which
237 had previously been reported as being removed during activation by host insect proteases presented
238 a dilemma. We initially speculated that the gut proteases of *A. aegypti* could not remove this region.
239 Tests showed that although the mosquitocidal enzymes did not remove as much of the protein as
240 had been reported for several lepidopteran species, it seemed likely that cleavage beyond the
241 specificity motifs had occurred. That observation led us to test the hypothesis that although cleavage
242 had taken place downstream of the specificity motifs that the N-terminal fragment remained
243 attached, non-covalently, to the rest of the protein and could participate in receptor binding. This
244 hypothesis was tested and found to be false, at least under the conditions of our assay the N-
245 terminus did not remain attached. The finding that when this N-terminal region was removed either
246 by proteolytic cleavage or deletion then activity to *A. aegypti* was lost confirmed the importance of
247 this region but further confused our understanding of its role. We don't currently have an
248 explanation for the dilemma, although could speculate that within the mosquito there might be a
249 proportion of Cry protein which is not proteolytically cleaved following ingestion and that this
250 fraction of the ingested protein could be responsible for the observed toxicity. Where individual *B.*
251 *thuringiensis* strains express multiple pesticidal proteins those proteins tend to show activity
252 towards the same type of insect, for example the Cry proteins expressed by Bt subsp *israelensis* and
253 Bt subsp *jegathesan* all have mosquitocidal activity (Zhang et al., 2017). Cry2Aa has generally been
254 isolated from strains with predominantly lepidopteran activity such as HD1 (Widner and Whiteley,
255 1989) where there is less evolutionary pressure to retain efficient activity against a mosquito.

256 **Funding**

257 Lazarus Joseph Goje acknowledges the Nigerian Tertiary Education Trust Fund for supporting his PhD
258 Scholarship.

259

260 **References**

261

262 Abdullah, M. A., et al., 2003. Introduction of Culex toxicity into *Bacillus thuringiensis* Cry4Ba by
263 protein engineering. Appl Environ Microbiol. 69**,** 5343-53.
264 Audtho, M., et al., 1999. Production of chymotrypsin-resistant *Bacillus thuringiensis* Cry2Aa1 delta-
265 endotoxin by protein engineering. Appl Environ Microbiol. 65**,** 4601-5.
266 Badran, A. H., et al., 2016. Continuous evolution of Bacillus thuringiensis toxins overcomes insect
267 resistance. Nature. 533**,** 58-63.
268 Crickmore, N., Ellar, D. J., 1992. Involvement of a possible chaperonin in the efficient expression of a
269 cloned CryIIA delta-endotoxin gene in *Bacillus thuringiensis*. Mol Microbiol. 6**,** 1533-7.
270 Crickmore, N., et al., 1994. Use of an operon fusion to induce expression and crystallisation of a
271 *Bacillus thuringiensis* delta-endotoxin encoded by a cryptic gene. Mol Gen Genet. 242**,** 365-
272 8.
273 Donovan, W. P., et al., 1988. Amino acid sequence and entomocidal activity of the P2 crystal protein.
274 An insect toxin from Bacillus thuringiensis var. kurstaki. J Biol Chem. 263**,** 561-7.
275 Jurat-Fuentes, J. L., Crickmore, N., 2017. Specificity determinants for Cry insecticidal proteins:
276 Insights from their mode of action. J Invertebr Pathol. 142**,** 5-10.
277 Kelley, L. A., et al., 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat
278 Protoc. 10**,** 845-58.
279 Liang, Y., Dean, D. H., 1994. Location of a lepidopteran specificity region in insecticidal crystal protein
280 CryIIA from *Bacillus thuringiensis*. Mol Microbiol. 13**,** 569-75.
281 Liu, X. S., Dean, D. H., 2006. Redesigning *Bacillus thuringiensis* Cry1Aa toxin into a mosquito toxin.
282 Protein Eng Des Sel. 19**,** 107-11.
283 Mandal, C. C., et al., 2007. Prediction-based protein engineering of domain I of Cry2A entomocidal
284 toxin of *Bacillus thuringiensis* for the enhancement of toxicity against lepidopteran insects.
285 Protein Eng Des Sel. 20**,** 599-606.
286 McNeil, B. C., Dean, D. H., 2011. *Bacillus thuringiensis* Cry2Ab is active on *Anopheles* mosquitoes:
287 single D block exchanges reveal critical residues involved in activity. FEMS Microbiol Lett.
288 325**,** 16-21.
289 Morse, R. J., et al., 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope.
290 Structure. 9**,** 409-17.
291 Ohsawa, M., et al., 2012. A 50-kilodalton Cry2A peptide is lethal to Bombyx mori and Lymantria
292 dispar. Appl Environ Microbiol. 78**,** 4755-7.
293 Shu, C., et al., 2017. A natural hybrid of a *Bacillus thuringiensis* Cry2A toxin implicates Domain I in
294 specificity determination. J Invertebr Pathol. 150**,** 35-40.
295 van Frankenhuyzen, K., 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J Invertebr
296 Pathol. 101**,** 1-16.
297 van Frankenhuyzen, K., 2013. Cross-order and cross-phylum activity of *Bacillus thuringiensis*
298 pesticidal proteins. J Invertebr Pathol. 114**,** 76-85.
299 WHO, Guidelines for laboratory and field testing of mosquito larvicides.
300 https://apps.who.int/iris/bitstream/handle/10665/69101/WHO_CDS_WHOPES_GCDPP_200
301 5.13.pdf. 2005.
302 Widner, W. R., Whiteley, H. R., 1989. Two highly related insecticidal crystal proteins of *Bacillus*
303 *thuringiensis* subsp. *kurstaki* possess different host range specificities. J Bacteriol. 171**,** 965-
304 74.
305 Widner, W. R., Whiteley, H. R., 1990. Location of the dipteran specificity region in a lepidopteran-
306 dipteran crystal protein from *Bacillus thuringiensis*. J Bacteriol. 172**,** 2826-32.
307 Xu, L., et al., 2016. Proteolytic Activation of *Bacillus thuringiensis* Cry2Ab through a Belt-and-Braces
308 Approach. J Agric Food Chem. 64**,** 7195-200.
309 Zhang, Q., et al., 2017. Effects and mechanisms of *Bacillus thuringiensis* crystal toxins for mosquito
310 larvae. Insect Sci. 24**,** 714-729.

311 Table 1 List of Cry2 proteins and their derivatives used in this study and their toxicity to *Aedes*
312 *aegypti*.

Cry2 protein (accession no.)	**Source strain**	**Toxicity to *A. aegypti***	**% mortality**
Cry2Aa2 (AAA83516)	HD1	Yes	65%
Cry2Aa17 (AIS85927)	LS5115-3	No	<10%
Cry2Ab4 (AAO13296)	B-Pr-88	No	<10%
Cry2Ab1 (AAA22342)	HD1	No	<10%
Cry2Ab29 (AIS85926)	DS415	No	<10%
Cry2Ac11 (CAM83895)	HD29	Yes	50%
Cry2Ad5 (CAO78739)	HD29	No	<10%
Cry2Ah1 (ACL13555)	SC6H8	No	<10%
Cry2Am1 (AIT70915)	Pooled genomes	Yes	20%
Cry2 Ac Ab Ab	This study	Yes	30%
Cry2 Ab Ac Ac	This study	No	<10%
Cry2 Ab(NT) Aa Aa	This study	No	<10%
Cry2 Aa(NT) Ab Ab	This study	Yes	55%
Cry2 Ab(NT) Ac Ac	This study	No	<10%
Cry2 Ac(NT) Ab Ab	This study	Yes	40%
Cry2Ab Q27E	This study	No	<10%
Cry2Ab KNN43-45RTD	This study	No	<10%
Cry2Ab Q27E KNN43-45RTD	This study	Yes	60%
Cry2Aa E27Q	This study	No	<10%
Cry2Aa RTD43-45KNN	This study	No	<10%
Cry2Ab Q27E KNN43-45RNN	This study	No	<10%
Cry2Ab Q27E KNN43-45KTN	This study	No	<10%
Cry2Ab Q27E KNN43-45KND	This study	No	<10%
Cry2Ab Q27E KNN43-45RTN	This study	No	<10%
Cry2Ab Q27E KNN43-45RND	This study	No	<10%
Cry2Ab Q27E KNN43-45KTD	This study	No	<10%
Cry2Aa Del1-45	This study	No	<10%

313

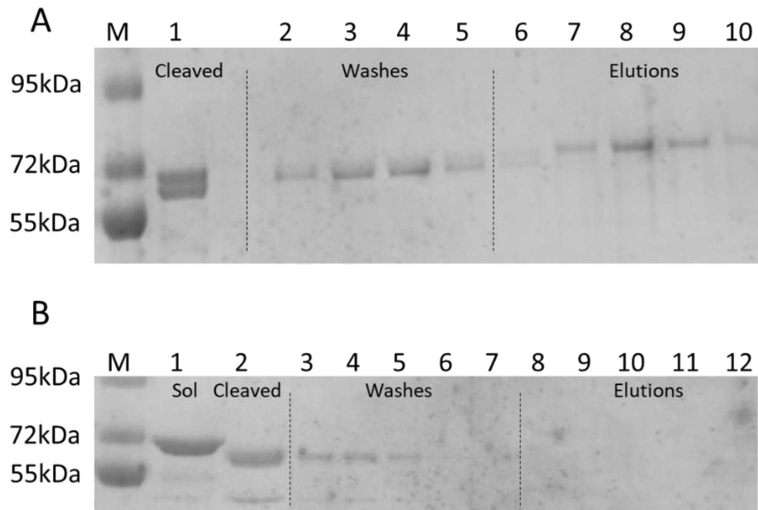
314

Fig. 1 Alignment of Cry2A proteins toxic (rows 1-3) or non-toxic (rows 4-9) to *Aedes aegypti*. Amino acids which correlate with toxicity are highlighted by the + symbol.

```
Cry2Aa2       MNNVLNSGRTTICDAYNVVAHDPFSFEHKSLDTIQKEWMEWKRTDHSLY
Cry2Ac11      MNTVLNNGRNTTCHAHNVVAHDPFSFEHKSLNTIEKEWKEWKRTDHSLY
Cry2Am1       MNNALNSGRTTICDAYNVVAHDPFSFEHKSLDTIQKEWMEWKRTDHSLY
                                        +               +++
Cry2Ah1       MNNVLNSGRATNGDAYNVVAHDPFSFQHKSLDTIQEEWMEWKKDNHILY
Cry2Ad5       MNSVLNSGRTTICDAYNVAAHDPFSFQHKSLDTVQKEWTEWKKNNHSLY
Cry2Ab29      MNSVLNSGRTTICDAYNVAAHDPFSFQHKSLDTVQKEWTEWKKNNHSLY
Cry2Ab1       MNSVLNSGRTTICDAYNVAAHDPFSFQHKSLDTVQKEWTEWKKNNHSLY
Cry2Ab4       MNSVLNSGRTTICDAYNVAAHDPFSFQHKSLDTVQKEWTEWKKNNHSLY
Cry2Aa17      MNSVLNSGRTTICDAYNVAAHDPFSFQHKSLDTVQKEWTEWKKNNHSLY
```

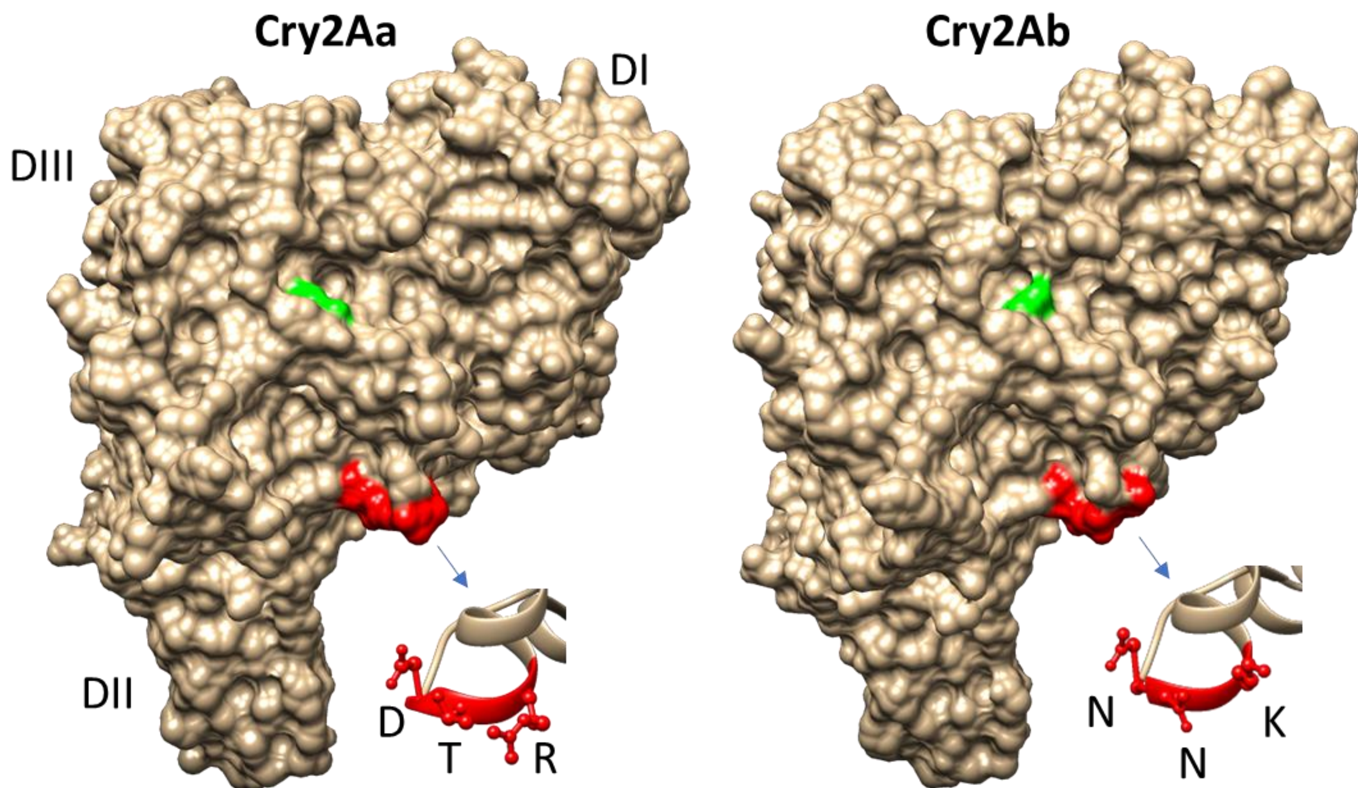
Fig. 2 Cleavage of Cry2Aa with chymotrypsin and *A. aegypti* gut extract. A) lane 1 crude *E. coli* derived Cry2Aa sample; lanes 2-4 digestion of Cry2Aa with 2mg/ml, 0.2mg/ml and 0.02mg/ml chymotrypsin respectively. B) lane 1 solubilized *B. thuringiensis* derived Cry2Aa; lanes 2-5 digestion of Cry2Aa with 8mg/ml, 4mg/ml, 0.4mg/ml and 0.04mg/ml chymotrypsin respectively; lanes 6-7 digestion of Cry2Aa with 1 in 20, 1 in 100 and 1 in 200 dilution of *A. aegypti* gut extract respectively.

353 Fig. 3 Attachment of His-tagged cleaved and uncleaved Cry2Aa to Ni-NTA beads. A) lane 1 partial
354 digest of Cry2Aa with chymotrypsin; lanes 2-3 washes from Ni-NTA bead column; lanes 6-10 elutions
355 from column with 150mM imidazole. B) lane 1 solubilized Cry2Aa; lane 2 Cry2Aa cleaved with *A.*
356 *aegypti* gut extract; lanes 3-7 washes from Ni-NTA bead column; lanes 8-12 elutions from column
357 with 150mM imidazole.
358



359

360 Fig. 4 Comparison of *A. aegypti* specificity motifs in Cry2Aa and Cry2Ab. The Cry2Aa structure was
361 determined experimentally (Morse et al., 2001) and that of Cry2Ab using Phyre2 modelling (Kelley et
362 al., 2015). The amino acids associated with *A. aegypti* specificity are highlighted by colour and the
363 inserts show ball and stick representations of the 43-45 triads.
364



365
366

367 Supplementary Table 1. Primers used in this study

Line	Primer name	Sequence	5'-Pho
368	Primer name	Sequence	5’-Pho
369			
370	Domain I swap		
371	Cry2A DI_F	ATGAATAATGTATTGAATAAYGGAAG	Yes
372	Cry2A DI_R	TTTAAATAACGACCAGATRGAKACATA	Yes
373	Cry2A w/o DI_F	TATCAAAGCCTTCTAGTATCTTCYG	No
374	Cry2A w/o DI_R	ATAAAATTCCTCCTTAATCGAATTC	No
375			
376	N-terminal swap		
377	Cry2A N-term_F	GCTGCGCGTAACCACCAC	Yes
378	Cry2Aa N-term_R	TAAACTATGATTCTGTTCTTTTCCACTC	Yes
379	Cry2Ab N-term_R	TAAACTATGATATTTTTTTCCACTCC	Yes
380	Cry2Ac N-term_R	TAAACTATGATCAGTTCTTTTCCATTC	Yes
381	Cry2A w/o N-term_R	GTGACCGCTACACTTGCCAG	No
382	Cry2Ac-w/o N-term_F	TATGTAGCCCCTATTGTGGGAAC	No
383	Cry2Ab w/o N-term_F	TACCTAGATCCTATTGTTGGAACTGTG	No
384			
385	N-terminal deletion		
386	Del45_F	CATAGTTTATATGTAGCTCCTGTAG	Yes
387	Del45_R	CATATAAAATTCCTCCTTAATCG	Yes
388			
389	Mutations		
390	Cry2Ab-RTD_F	CGTACCGATCATAGTTTATACCTAGATCC	Yes
391	Cry2Ab-RTD_R	TTTCCACTCCGTCCATTCC	Yes
392	Cry2Ab-E_F	GAACACAAATCATTAGATACCGTAC	Yes
393	Cry2Ab-E_R	AAAACTAAATGGATCATGAGA	Yes
394	Cry2Aa-Q_F	CAACATAAATCATTAGATACCATCC	Yes
395	Cry2Aa-Q_R	AAAACTAAATGGATCATGGG	Yes
396	Cry2Aa-KNN_F	AATAATCATAGTTTATATGTAGCTCC	Yes
397	Cry2Aa-KNN_R	TTTTTTCCACTCATCCATTCTTTTTGG	Yes
398	Cry2Ab-ERNN_F	AATAATCATAGTTTATATATGTAGCTCC	Yes
399	Cry2Ab-ERNN_R	TCTTTTCCACTCCATCCATTCTTTTTGG	Yes
400	Cry2Ab-EKTN_F	ACTAATCATAGTTTATATGTAGCTCC	Yes
401	Cry2Ab-EKTN_R	TTTTTTCCACTCCATTCCATTCTTTTTGG	Yes
402	Cry2Ab-EKND_F	AATGATCATAGTTTATATGTAGCTCC	Yes
403	Cry2Ab-EKND_R	TTTTTTCCACTCCATCCATTCTTTTTGG	Yes
404	Cry2Ab-ERTN_F	ACTAATCATAGTTTATATGTTAGCTCC	Yes
405	Cry2Ab-ERTN_R	TCTTTTCCACTCCATCCATTCTTTTTGG	Yes
406	Cry2Ab-ERND_F	AATGATCATAGTTTATATGTAGCTCC	Yes
407	Cry2Ab-ERND_R	TCTTTTCCACTCCATCCATTCTTTTTGG	Yes
408	Cry2Ab-EKTD_F	AAAACTGATCATAGTTTATACCTAGATC	Yes
409	Cry2Ab-EKTD_R	TTTTTTCCACTCCATCCATTCTTTTTGG	Yes
410	Cry2Aa L144A_F	CCCTGTTCCTGCATCAATAACTTC	Yes
411	Cry2Aa L144A_R	TTTTGAGTAGGGTTTAAAAAATTATC	Yes
412			
413	His tag addition		
414	Cry2AaHis_F	CATCATCACCATCATCACAATAATGTATTGAATAGTGG	Yes
415	Cry2AaHis_R	CATATAAAATTCCTCCTTAAATATC	Yes
416			