Channel-pore cation selectivity is a major determinant of *Bacillus thuringiensis* Cry46Ab mosquitocidal activity.

Tohru Hayakawa*, Midoka Miyazaki, Syoya Harada, Mami Asakura, Toru Ide

Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan

***Corresponding author**

Tohru Hayakawa, Email address: hayaka-t@cc.okayama-u.ac.jp

Abstract

Cry46Ab from *Bacillus thuringiensis* TK-E6 is a new mosquitocidal toxin with an aerolysin-type architecture, and it is expected to be used as a novel bioinsecticide. Cry46Ab acts as a functional pore-forming toxin, and characteristics of the resulting channel-pores, including ion selectivity, have been analyzed. However, the relationship between channel-pore ion selectivity and insecticidal activity remains to be elucidated. To clarify the effects of charged amino acid residues on the ion permeability of channel-pores and the resulting insecticidal activity, in the present study, we constructed Cry46Ab mutants in which a charged amino acid residue within a putative transmembrane β-hairpin region was replaced with an oppositely charged residue. Bioassays using *Culex pipiens* mosquito larvae revealed that the mosquitocidal activity was altered by the mutation. A K155E Cry46Ab mutant exhibited toxicity apparently higher than that of wild-type Cry46Ab, but the E159K and E163K mutants exhibited decreased toxicity. Ions selectivity measurements demonstrated that the channel-pores formed by both wild-type and mutant Cry46Abs were cation selective, and their cation preference was also similar. However, the degree of cation selectivity was apparently higher in channel-pores formed by the K155E mutant, and reduced selectivity was observed with the E159K and E163K mutants. Our data suggest that channel-pore cation selectivity is a major determinant of Cry46Ab mosquitocidal activity and that cation selectivity can be controlled via mutagenesis targeting the transmembrane β-hairpin region.

Key points

1. Cry46Ab mutants were constructed by targeting the putative transmembrane β-hairpin region.
2. Charged residues within the β-hairpin control the flux of ions through channel-pores.
3. Channel-pore cation selectivity is correlated with insecticidal activity.

Keywords

Bacillus thuringiensis TK-E6; Cry46Ab toxin; *Culex pipiens* mosquito larvae; site-directed mutagenesis; electrophysiologic analysis

Introduction

Cry46Ab is a crystal protein derived from *Bacillus thuringiensis* strain TK-E6. Cry46Ab has recently renamed as Mpp46Ab1 in new nomenclature (Crickmore et al. 2020). It has been shown that upon activation by proteinase K, Cry46Ab is highly cytotoxic to human leukemic T cells (MOLT-4 and Jurkat), but has virtually no effect on human embryonic kidney cells (HEK293). Cry46Ab was therefore designated parasporin 2Ab, a member of a family of toxins that exhibit preferential cytotoxicity against human cancer cells (Hayakawa et al. 2007). In addition, it was recently reported that Cry46Ab exhibits apparent insecticidal activity against larvae of the mosquito *Culex pipiens* (Hayakawa et al. 2017). It is noteworthy that co-administration of Cry46Ab with other mosquitocidal Cry toxins, particularly the combination of Cry46Ab and Cry4Aa from *B. thuringiensis* subsp. *israelensis*, results in significant synergistic toxicity against *C. pipiens* larvae (Hayakawa et al. 2017). Cry46Ab is therefore expected to be used not only as a new type of bioinsecticide but also as an agent to enhance the mosquitocidal activity of other Cry toxins.

Cry46Ab exhibits significant homology (84% identity) to Cry46Aa (designated parasporin-2Aa) from *B. thuringiensis* strain A1547 (Hayakawa et al. 2007). Although Cry46Aa is cytotoxic to human leukemic T cells, no insecticidal activity has been reported (Kim et al. 2000). X-ray crystallography analyses revealed that the three-dimensional structure of Cry46Aa is similar to that of aerolysin-type β pore-forming toxins (β-PFTs) (Akiba et al. 2009). Based on its high degree of homology with Cry46Aa, Cry46Ab is thought to be a member of the aerolysin-type β-PFT family (Hayakawa et al. 2007). Previous studies demonstrated that Cry46Ab functions as a PFT, producing cation-selective channel-pores in artificial lipid bilayers (Hayakawa et al. 2017; Sakakibara et al. 2019). The reported cation preference of the channel-pores is generally $K^+ > Na^+$, $K^+ > Ca^{2+}$, and $Ca^{2+} > Na^+$ (Sakakibara et al. 2019).

Intriguingly, Cry46Ab does not exhibit homology to most other Cry toxins. Indeed, nearly 90% of Cry toxins share a characteristic three-domain architecture (domains I, II, and III) and form a large homology group (Schnepf et al. 1998). In general, domain I is located in the N-terminal region and consists of a bundle of seven amphipathic α-helices. The α-helices of domain I are thought to

form a transmembrane pore, and therefore, these three-domain Cry toxins are classified as α-PFTs. Domain II, which consists of three antiparallel β-sheets, is a putative receptor-binding domain. Domain III, located in the C-terminal region, contains two antiparallel β-sheets that form a β-sandwich fold with a jellyroll topology. Domain III is assumed to be involved in controlling structural integrity and/or receptor binding (Schnepf et al. 1998). Thus, despite the differences in their structures, both aerolysin-type Cry46Ab and three-domain Cry toxins are thought to function via a similar insecticidal mechanism involving pore formation. According to the colloid-osmotic lysis model, pores formed by Cry toxins allow ions and water to pass into target cells, resulting in disruption of the membrane potential, followed by swelling, lysis, and the eventual death of the host cell (Knowles 1994; Knowles and Ellar 1987). On the other hand, the characteristics of the channel-pores formed by Cry toxins have not been investigated in detail. Furthermore, the correlation between channel-pore formation and insecticidal activity is not fully understood.

A β-hairpin structure in the middle domain is a characteristic of aerolysin-type β-PFTs. Similar structures have been found in many aerolysin-type β-PFTs, such as aerolysin (Iacovache et al. 2006), staphylococcal α-toxin (Song et al. 1996), enterotoxin from *Clostridium perfringens* (Kitadokoro et al. 2011), ε-toxin from *C. perfringens* (Cole et al. 2004), hemolytic lectin from parasitic mushroom *Laetiporus sulphureus* (Mancheño et al. 2004), leukocidin (Miles et al. 2002), and Cry46Aa (Akiba et al. 2009). According to the pore-formation model of aerolysin, after binding to glycosylphosphatidylinositol-anchored receptor proteins on the target cell membrane, the β-hairpin inserts into the membrane and rearranges into a transmembrane β-barrel (Degiacomi et al. 2013; Xu et al. 2014; Rossjohn et al. 1998). In general, the β-hairpin is composed of an alternating pattern of polar and hydrophobic amino acid residues, suggesting that it is amphipathic. The polar and hydrophobic residues are believed to face the hydrophilic lumen and lipid bilayer of the transmembrane β-barrel, respectively. It has been proposed that the charged amino acid residues within the transmembrane β-hairpin control the flux of ions through the channel-pores (Benz and Popoff 2018). Indeed, the transmembrane β-hairpin of aerolysin contains an excess of positively charged residues (four lysine residues and three glutamic acid residues) and forms anion-selective

channel-pores (Chakraborty et al. 1990). Similarly, the corresponding region of *C. perfringens* ε-toxin contains an excess of positively charged residues (one lysine residue and no negatively charged residues) and forms anion-selective channel-pores (Petit et al. 2001). In contrast, the β-hairpin region of *C. perfringens* enterotoxin contains an excess of negatively charged amino acid residues (no positively charged residues and three glutamic acid residues) and forms cation-selective channel-pores (Kitadokoro et al. 2011).

In the present study, we predicted the transmembrane β-hairpin region of Cry46Ab based on sequence alignment analysis with the closely related Cry46Aa, and constructed four Cry46Ab mutants (K155E, K156E, E159K, and E163K) one of the charged amino acid residue in the putative transmembrane region was replaced with an oppositely charged residue. These charged amino acid residues were assumed to line the lumen side of the channel-pores and thus affect the ion permeability of the pores. To investigate the effect of the charged amino acid residues in the transmembrane domain of Cry46Ab on channel-pore ion permeability and clarify the relationship between channel-pore ion permeability and insecticidal activity, the Cry46Ab mutants were subjected to bioassays using *Culex pipiens* mosquito larvae and ion-selectivity measurements using planar lipid bilayers.

Materials and methods

Construction of the Cry46Ab mutants

In the structural model of Cry46Aa, which is most closely related to Cry46Ab, the transmembrane domain is thought to be a β-hairpin region consisting of β8-loop-β9 (Fig. 1a, Akiba et al. 2009). The corresponding region in Cry46Ab spans residues L^{152} to T^{168} and contains two positively charged lysine residues (K^{155} and K^{156}) and two negatively charged glutamic acid residues (E^{159} and E^{163}) (Fig. 1b).

To investigate effect of these charged amino acids on the ion permeability of channel-pores formed by Cry46Ab and on the insecticidal activity resulting from the formation of channel-pores by Cry46Ab, in the present study, we constructed four Cry46Ab substitution mutants (K155E, K156E,

E159K, and E163K). In these mutants, one charged amino acid was replaced with an oppositely charged amino acid (Fig. 1b). The mutations were introduced via site-directed mutagenesis, as reported previously (Howlader et al. 2009). The expression vector, pGST-Cry46Ab-S1 (Hayakawa et al. 2017) was used as a template. The primers used for mutagenesis are listed in Table 1. Introduction of the desired mutations was confirmed by DNA sequencing.

Preparation of Cry46Ab toxins

Wild-type and mutant Cry46Abs were expressed as glutathione *S*-transferase (GST) fusions in *Escherichia coli* BL21 and purified as described previously (Hayakawa et al. 2017). Briefly, *E. coli* cells were cultured in TB medium containing ampicillin (100 μg/mL) until the OD_{600} reached 0.5-0.7, and then expression of the GST-Cry46Abs was induced by incubation in 0.1 mM isopropyl-β-$_D$-thiogalactopyranoside at 30°C for 4 h. The *E. coli* cells were harvested by centrifugation and then disrupted by sonication, and the GST-Cry46Abs were purified using glutathione-Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. The GST-Cry46Abs were then activated by passage through an immobilized-trypsin column prepared as described previously (Hayakawa et al. 2017). The activated Cry46Abs (polypeptides of 29 kDa) were concentrated using Vivaspin 6 (10-kDa MWCO) centrifugal filter devices (GE Healthcare, Little Chalfont, UK). Protein concentration was estimated using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as the standard, and the purified peptides were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Measurement of mosquito-larvicidal activity

To determine the mosquito-larvicidal activity, purified GST-Cry46Ab wild-type and mutants were administered to *Culex pipiens* larvae (3rd instar) as described previously (Hayakawa et al. 2017). Mosquito larvae were reared from eggs supplied by the Research and Development Laboratory, Dainihon Jochugiku Co., Ltd. (Osaka, Japan). Mortality was recorded 48 h after toxin addition. The experiments were repeated three times independently, and the average and standard deviation of the

mortality data were calculated. The 50% lethal dose (LC_{50}) was determined using PROBIT analysis (Finney 1971).

Ion selectivity measurements

Characteristics of the channel-pores formed by Cry46Abs were analyzed using a previously described instrument (Hayakawa et al. 2017; Sakakibara et al. 2019). Briefly, the instrument consisted of two chambers (upper, *cis* chamber; lower, *trans* chamber), and both chambers were held at virtual ground, such that the voltage in the solution of the *cis* chamber was connected to a patch-clamp amplifier by an Ag/AgCl electrode-defined membrane potential. The bottom of the *cis* chamber was a thin sheet of polyvinyl chloride with a small circular hole (approximately φ200 μm), and a lipid bilayer was constructed by painting asolectin (phospholipids from soybean, Sigma-Aldrich Corp.) solution (40 mg/mL in n-decane) across the small hole. At the same time, liposomes consisting of asolectin were prepared in solution containing 1 M sucrose, as described previously (Sakakibara et al. 2019).

To constitute Cry46Ab channel-pores in the lipid bilayer, trypsin-activated Cry46Abs were mixed with liposome solution at a concentration of 25 μg/mL. A previous study suggested that the channel-pores formed by Cry46Ab affect the integrity of lipid bilayer membranes and disrupt liposomes (Sakakibara et al. 2019). The mixture (Cry46Ab and liposome) was added to the solution in the *cis* chamber to facilitate fusion between the liposomes and the planar lipid bilayer. To analyze anion-cation selectivity of channel-pores formed by Cry46Abs, membrane currents were recorded using a 4-fold gradient of KCl across the lipid bilayer (600 mM KCl and 10 mM Tris-HCl [pH 8.0] in the *cis* chamber, 150 mM KCl and 10 mM Tris-HCl [pH 8.0] in the *trans* chamber). In addition, to analyze cation preference (K^+, Na^+, or Ca^{2+}) of channel-pores, different salt solutions (KCl, NaCl, or $CaCl_2$) were used in the *cis* and *trans* chambers. KCl and NaCl were used at a concentration of 150 mM, and $CaCl_2$ was used at a concentration of 75 mM to provide Cl^- at a concentration equivalent to that in the KCl and NaCl solutions. Data were analyzed using pClamp software (Axon Instruments, Roster City, CA). The current amplitude of the resolvable steps was recorded for each experiment,

and the resulting data were plotted versus the corresponding applied voltage to generate current-voltage relationships. The zero-current reversal potential (V_R) was obtained as the X-intercept of the current-voltage relationship line. The permeability ratio was calculated using the Goldman-Hodgkin-Katz equation.

Results

Preparation of wild-type and mutant Cry46Abs

Wild-type and mutant Cry46Abs were expressed as GST fusions in *E. coli*. SDS-PAGE analysis indicated that the molecular mass of the purified GST-Cry46Abs was approximately 60 kDa, very similar to the expected mass (59.309 kDa) (Fig. 2a). In addition, several protein bands of higher molecular mass suggestive of homodimer formation were observed, particularly in the wild-type GST-Cry46Ab and K155E and K156E mutant samples (Fig. 2a).

The wild-type and mutant GST-Cry46Abs were then subjected to treatment using an immobilized-trypsin column. As Cry toxins are activated by trypsin-like proteases in the midgut juice of susceptible insect larvae, this assay serves as a presumptive test of folding fidelity (Almond and Dean 1993). SDS-PAGE analysis revealed that wild-type GST-Cry46Ab was quickly (within 15 min) processed into a polypeptide of 29 kDa by this treatment (Fig. 2b). The 29 kDa polypeptide was very similar in size to activated Cry46Ab as reported previously (Hayakawa et al. 2007; 2017) and remained stable for at least 30 min (Fig. 2b). The K155E, E159K, and E163K mutants exhibited a processing pattern very similar to that of wild-type Cry46Ab, suggesting high folding fidelity compared with the wild type (Fig. 2b). However, the K156E mutant was apparently over-processed, such that the amount of 29-kDa polypeptide remaining after 15 min was undetectable by SDS-PAGE (Fig. 2b). This suggested that folding fidelity of overall toxin structure was disrupted by replacement of K^{156} with E.

Mosquitocidal activity of wild-type and mutant Cry46Abs

The mosquitocidal activity of the wild-type and mutant GST-Cry46Abs was assayed using *C. pipiens*

larvae. Purified GST was used as a negative control and exhibited no toxicity at concentrations up to 2 μg/mL (data not shown). Wild-type Cry46Ab exhibited toxicity against *C. pipiens* larvae, with an LC_{50} value (95% confidence limits) of 0.98 (0.95-1.02) μg/mL (Fig. 3). This LC_{50} value was very similar to that (1.02 μg/mL) reported previously (Hayakawa et al. 2017).

Interestingly, the K155E mutant exhibited toxicity apparently higher than that of wild-type Cry46Ab, with an LC_{50} value (95% confidence limits) of 0.54 (0.52-0.56) μg/mL (Fig. 3). The remaining mutants, particularly the E163K mutant, exhibited lower toxicity against *C. pipiens* larvae compared with the wild type. The LC_{50} values (95% confidence limits) for the K156E, E159K, and E163K mutants were 1.90 (1.80-2.01), 1.53 (1.45-1.63), and 2.74 (2.50-3.06) μg/mL, respectively (Fig. 3). In the case of the K156E mutant, excessive degradation in the midgut juice of *C. pipiens* larvae was thought to be responsible for the lower toxicity. Therefore, the K156E mutant was not subjected to further analysis. In contrast, the remaining mutants (K155E, E159K, and E163K) exhibited stability upon trypsin treatment comparable to that of wild-type Cry46Ab (Fig. 2B), suggesting that the observed difference in toxicity was due to changes in one or more characteristics of the channel-pores.

Anion-cation selectivity of channel-pores formed by wild-type Cry46Ab

Interestingly, two different current amplitudes were observed in the measurements. One current amplitude was similar to those observed previously (Hayakawa et al. 2017; Sakakibara et al. 2019), characterized as a rapid flickering between open and closed states (Fig. 4a). In previous measurements, activated wild-type Cry46Ab was added directly to the solution in the *cis* chamber, and the current amplitude of this type was thought to be generated by channel-pores that were directly constituted in the planar lipid bilayer. The current amplitude of the resolvable steps was recoded, pooled for seven independent experiments, and plotted versus the corresponding applied voltage to generate a current-voltage relationship. The current-voltage relationship was a linear, and the channel conductance and V_R value were 750 pS and −11.82 mV, respectively (Fig. 4b). The P_K/P_{Cl} permeability ratio calculated from this V_R value was 2.21, demonstrating a higher

permeability for K^+ than Cl^-. Formation of cation-selective channel-pores by wild-type Cry46Ab was observed in previous measurements (Sakakibara et al. 2019).

The second type of current amplitude was very stable, remaining in the open state for at least several minutes (Fig. 4c). This type of current amplitude was much larger than that described above (Fig. 4a and c), suggesting that multiple channel-pores were formed in the planar lipid bilayer. After the formation of channel-pores in the liposomes, only those liposomes that were destabilized by the formation of multiple channel-pores seemed to fuse with the planar membrane. The current-voltage relationship was a linear, with different conductance levels (ranging from 3.40 to 5.32 nS) in each measurement (Fig. 4d). This suggested that the number of channel-pores formed in the liposomes varied in each measurement. The V_R value was −9.38 ± 0.66 mV (n = 7 independent measurements), and the P_K/P_{Cl} permeability ratio calculated from this V_R value was 1.86, demonstrating a higher permeability for K^+ than Cl^-. Because the V_R values were very similar for both types of current amplitudes, these current amplitudes were thought to be generated by the same type of channel-pores. The current amplitudes that stably remained in the open state were subjected to further analysis using channel-pores formed by the Cry46Ab mutants.

Anion-cation selectivity of channel-pores formed by Cry46Ab mutants

Membrane currents through the channel-pores formed by Cry46Ab mutants were recorded as conducted for wild-type Cry46Ab and plotted versus the corresponding applied voltage. The current-voltage relationships for the channel-pores formed by the mutants were linear, with different conductance levels in each measurement (Fig. 5).

The V_R values obtained with the Cry46Ab mutants varied. The V_R value for channel-pores formed by the K155E mutant was −17,06 ± 2.82 mV (n = 5), with a calculated P_K/P_{Cl} permeability ratio of 3.29 (Fig. 5). This P_K/P_{Cl} permeability ratio was apparently greater than that of wild-type Cry46Ab, suggesting a much higher permeability for K^+ than Cl^-. Collectively, these data suggested that replacement of residue K^{155} with an E residue in the putative transmembrane domain of Cry46Ab increased the negative charge in the channel-pores, resulting in higher permeability for K^+

than Cl^-.

In contrast, the V_R values for the channel-pores formed by the E159K and E163K mutants were similar, at −6.17 ± 1.58 mV (n = 5) and −5.66 ± 2.30 mV (n = 11), respectively (Fig. 5). The calculated P_K/P_{Cl} permeability ratios for the E159K and E163K mutants were 1.50 and 1.45, respectively, slightly lower than that of wild-type Cry46Ab and significantly lower than that of the K155E mutant. This suggested that, contrary to the case of the K155E mutant, replacement of residues E^{159} and E^{163} with K residue in the putative transmembrane domain increased the positive charge in the channel-pores, resulting in limited permeability of K^+.

Cation preference

When the *cis* chamber contained 150 mM KCl and the *trans* chamber 150 mM NaCl, the V_R values for the channel-pores formed by wild-type Cry46Ab and the K155E, E159K, and E163K mutants were −10.51 ± 0.94 (n = 3), −4.09 ± 1.00 (n = 4), −2.54 ± 1.67 (n = 4), and −5.41 ± 2.12 mV (n = 3), respectively (Fig. 6). The P_K/P_{Na} permeability ratios were calculated using the above V_R values and corresponding P_K/P_{Cl} permeability ratios and determined to be 2.07 (wild-type), 1.24 (K155E), 1.19 (E159K), and 1.47 (E163K), respectively (Table 2). This indicated that the channel-pores formed by the wild-type and mutant Cry46Abs exhibit a preference for K^+ over Na^+. In addition, the mutations appeared to reduce the selectivity.

Similarly, when the *cis* chamber contained 75 mM $CaCl_2$ and the *trans* chamber 150 mM KCl, the V_R values for channel-pores formed by wild-type Cry46Ab and the K155E, E159K, and E163K mutants were 2.08 ± 0.84 (n = 3), 2.59 ± 0.63 (n = 3), 4.75 ± 0.62 (n = 3), and 3.79 ± 1.51 mV (n = 3), respectively (Fig. 7). The P_K/P_{Ca} permeability ratios were calculated using the above V_R values and corresponding P_K/P_{Cl} permeability ratios and determined to be 1.18 (wild-type), 1.27 (K155E), 1.52 (E159K), and 1.39 (E163K), respectively (Table 2). Thus, the wild-type and mutant Cry46Abs formed channel-pores in which the permeability of K^+ was slightly higher than that of Ca^{2+}.

When the *cis* chamber contained 75 mM $CaCl_2$ and the *trans* chamber 150 mM NaCl, the

284 V_R values for the channel-pores formed by wild-type Cry46Ab and the K155E, E159K, and E163K
285 mutants were −4.83 ± 0.95 (n = 3), −1.81 ± 1.23 (n = 3), −8.00 ± 1.09 (n = 3), and −1.45 ± 0.22 mV
286 (n = 3), respectively (Fig. 8). The P_{Na}/P_{Ca} permeability ratios were calculated using the above V_R
287 values and P_K/P_{Cl} and P_K/P_{Na} permeability ratios and determined to be 0.63 (wild-type), 0.88
288 (K155E), 0.51 (E159K), and 0.87 (E163K), respectively (Table 2). These data demonstrated that the
289 wild-type and mutant Cry46Abs formed channel-pores in which the permeability of Ca^{2+} was
290 slightly higher than that of Na^+.
291 Collectively, the above data indicate that there was no difference in the cation preference
292 (K^+, Na^+, or Ca^{2+}) of the channel-pores between the wild-type and mutant Cry46Abs. Although the
293 P_K/P_{Na}, P_K/P_{Ca}, and P_{Na}/P_{Ca} permeability ratios differed for some mutants, the differences were not
294 correlated with the differences in mosquitocidal activity.
295
296 **Discussion**
297 We previously demonstrated that Cry46Ab toxin forms cation-selective channel-pores in planar lipid
298 bilayer, and the characteristics of these channel-pores have been partially characterized (Hayakawa
299 et al. 2017; Sakakibara et al. 2019). In the mode of action of Cry46Ab, pore formation is thought to
300 be a central component, but the relationship between pore formation and the resulting insecticidal
301 activity remains to be elucidated. Therefore, we constructed substitution mutants (K155E, K156E,
302 E159K, and E163E) targeting the transmembrane β-hairpin region of Cry46Ab and investigated the
303 effects of these mutations on the selectivity of ions passing through the channel-pores and on the
304 resulting mosquitocidal activity. Based on sequence alignment analysis with the closely related
305 Cry46Aa (Fig. 1, Hayakawa et al. 2007), the transmembrane β-hairpin region of Cry46Ab was
306 hypothesized to span amino acid residues L^{152} to T^{168}. This region contains two positively charged
307 lysine residues (K^{155} and K^{156}) and two negatively charged glutamic acid residues (E^{159} and E^{163}),
308 and at least some of these residues are thought to face the hydrophilic lumen of the channel-pores
309 and thereby affect ion permeability.
310 Cry46Ab mutants (except K156E) were successfully expressed in *E. coli*. A bioassay using

311 *C. pipiens* mosquito larvae demonstrated that the toxicity of the K155E mutant (LC_{50} = 0.54 μg/mL)
312 was apparently higher than that of wild-type Cry46Ab (LC_{50} = 0.98 μg/mL). Ion selectivity
313 measurements demonstrated that the permeability ratio P_K/P_{Cl} of the channel-pores formed by the
314 K155E mutant (P_K/P_{Cl} = 3.29) was apparently higher than that of wild-type Cry46Ab (P_K/P_{Cl} = 1.86)
315 (Table 2). This suggests that, as expected for the K155E mutant, replacement of a positively charged
316 lysine (K^{155}) with a negatively charged glutamic acid (E) rendered the environment of the lumen
317 more anionic, resulting in the channel-pores becoming more cation selective. In contrast, the toxicity
318 of the E159K (LC_{50} = 1.53 μg/mL) and E163K (LC_{50} = 2.74 μg/mL) mutants was apparently lower
319 than that of wild-type Cry46Ab, and their P_K/P_{Cl} values (1.50 for E159K; 1.45 for E163K) were
320 slightly lower than that of wild-type Cry46Ab (Fig. 5; Table 2). This suggests that replacement of
321 negatively charged glutamic acid residues (E^{159} and E^{163}) with positively charged lysine residues (K)
322 rendered the environment of the lumen more cationic, resulting in the channel-pores becoming less
323 cation selective. Changing the P_K/P_{Cl} permeability ratio thus apparently affects mosquitocidal
324 activity, as increasing the selectivity of Cry46Ab resulted in higher toxicity against mosquito larvae.
325 The formation of highly cation-selective channel-pores may enhance the influx of cations and water
326 into the larval cell, thus disrupting the membrane potential and inducing swelling, lysis, and the
327 eventual death of the host cell. Nevertheless, this notion still needs to be elucidated. Further studies
328 using mutants combined either two of mutation K155E, E159K and E163E would be of great
329 interest. It would also be of interest to investigate mutants in which charged residues K155, E159
330 and E163 are replaced with other type of residue such as non-charged residues, and mutants in which
331 a polar residues other than K^{155}, E^{159} and E^{163} are replaced.
332 It has been demonstrated that co-administration of Cry46Ab with three-domain Cry toxins,
333 especially the combination of Cry46Ab and Cry4Aa, results in significant synergistic toxicity against
334 *C. pipiens* larvae (Hayakawa et al. 2017). It is believed that co-administration of multiple toxins
335 exhibiting different modes of action prevents the onset of resistance in insects. Synergistic toxicity is
336 observed when multiple toxins exhibiting different modes of action are co-administrated, suggesting
337 differences in the mode of action of Cry46Ab and three-domain Cry toxins. However, Cry46Ab has

been demonstrated to function as a PFT, and pore formation has also been demonstrated with several three-domain Cry toxins. According to the umbrella model, helices α4 and α5 of domain I insert into the membrane to form pores, while the remaining helices spread along the outer membrane surface via a conformational change (Gazit et al. 1998). As such, Cry46Ab and three-domain Cry toxins are thought to share a similar insecticidal mechanism based on pore formation. On the other hand, binding receptors seems to be different between aerolysin-type Cry toxins and three-domain Cry toxins (Xu et al. 2014), and the difference may proceed synergistic toxicity. However, the determinants that facilitate synergistic toxicity involving Cry46Ab and three-domain Cry toxins remain to be elucidated.

Interestingly, channel-pores formed by wild-type Cry46Ab exhibit a $K^+ > Na^+$, $K^+ > Ca^{2+}$, and $Ca^{2+} > Na^+$ cation preference (Sakakibara et al. 2019). In the present study, this observation was confirmed using a modified ion selectivity measurement procedure (Figs. 6-8). There was no significant difference in channel pore cation preference between the wild-type and mutant Cry46Abs. Although some of the mutations affected the P_K/P_{Na}, P_K/P_{Ca}, and P_{Na}/P_{Ca} values, the changes were not correlated with a change in mosquitocidal activity (Table 2). In contrast, it is widely accepted that the influx of ions into cells causes not only osmotic shock but also apoptosis, suggesting that differences in the cation preference of channel-pores may result in different effects on insecticidal activity. The characteristics of channel-pores have not been investigated in detail for three-domain Cry toxins. It would thus be of interest to investigate the cation preference of channel-pores formed by three-domain Cry toxins, especially Cry toxins that exhibit synergistic toxicity with Cry46Ab.

Contributions

TH and TI conceived and designed research. TH and SH constructed mutants and analyzed their biological activity. TH, MM, MA and TI contributed electrophysiologic experiments. TH, MM and TI analyzed data. TH wrote the manuscript. All authors read and approved the manuscript.

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461

462

463 Table 1. Nucleotide sequences of the primers used for site-directed mutagenesis.

Mutants	Primers	Primer sequence (5'→3')
K155E	46Ab-155E-f	GAAAAAGTCTTTGAAATTGGT
	46Ab-155-156r	AATCGACAGTTTAGTGGTAAT
K156E	46Ab156E-f	AAAGAAGTCTTTGAAATTGGT
	46Ab-155-156r	
E159K	46Ab159K-f	AAAATTGGTGGCGAAGTTTCG
	46Ab159-163r	AAAGACTTTTTTAATCGACAG
E163K	46Ab163K-f	GAAATTGGTGGCAAAGTTTCG
	46Ab159-163r	

464

465

Table 2. Relationship between mosquitocidal activity and ion permeability of channel-pores formed by wild-type and mutant Cry46Abs.

Cry46Ab	Mosquitocidal activity		Ion permeability ratios			
	LC_{50} (μg/mL)	95% confidence interval	P_K/P_{Cl}	P_K/P_{Na}	P_K/P_{Ca}	P_{Na}/P_{Ca}
WT	0.98	0.95 - 1.02	1.86	2.07	1.18	0.63
K155E	0.54	0.52 - 0.56	3.29	1.24	1.27	0.88
E159K	1.53	1.45 - 1.63	1.50	1.19	1.09	0.51
E163K	2.74	2.50 - 3.06	1.45	1.47	1.39	0.87

471 **Figure legends**

472

473 Fig. 1 Putative transmembrane β-hairpin region of Cry46Ab (A) Structural neighbors of Cry46Ab.
474 Ribbon diagrams are drawn from PDB data under codes 1PRE for aerolysin (Parker et al. 1994),
475 1UYJ for epsilon toxin (Cole et al. 2004), and 2ZTB for Cry46Aa (Akiba et al. 2009). All images for
476 the molecular structure is prepared with PyMOL. Putative transmembrane β-hairpin regions are
477 indicated by black color. (B) Comparison of the putative transmembrane β-hairpin regions of
478 different aerolysin-type β-PFTs. The alignment of aerolysin and epsilon toxin is adapted from
479 Iacovache et al. (2006). Cry46Aa (Kim et al. 2000) and Cry46Ab (Hayakawa et al. 2007) share
480 identical amino acid sequence in the putative transmembrane β-hairpin region. Amino acid residue
481 number is shown at left.

482

483 Fig. 2 Recombinant wild-type and mutant Cry46Abs. (A) Wild-type and mutant GST-Cry46Abs
484 were purified using glutathione beads and analyzed by 10% SDS-PAGE. One microgram of purified
485 protein was applied. (B) Wild-type and mutant Cry46Abs were treated with an immobilized-trypsin
486 column and analyzed by 15% SDS-PAGE.

487

488 Fig. 3 Mosquitocidal activity of wild-type and mutant GST-Cry46Abs. Filled circles, wild-type GST-
489 Cry46Ab; open circles, mutant GST-Cry46Abs. The experiments were repeated independently more
490 than three times. Average (standard deviation) mortality rates observed at 48 h after administration
491 are shown. The LC_{50} values (95% confidence limits) were determined using PROBIT analysis
492 (Finney, 1971).

493

494 Fig. 4 Anion-cation selectivity of channel-pores formed by wild-type Cry46Ab. Membrane currents
495 though the channel-pores formed by wild-type Cry46Ab were recorded with a 4-fold gradient of KCl
496 across the lipid bilayer. (A) Representative segments of membrane current flickering between open
497 and closed states. Current levels corresponding to the open state of the channel-pores are indicated

498 by a dashed line. (B) Current-voltage relationship of membrane current flickering between open and
499 closed states. The zero-current reversal potential (V_R) was calculated from the equation of the fitted
500 line. (C) Representative segments of membrane current remain in the open state for an extended
501 time. (D) The current-voltage relationship of membrane current remains in the open state for an
502 extended time. The experiment was repeated 7 times independently, and the average (standard
503 deviation) V_R was determined using each fitted line.
504
505 Fig. 5 Anion-cation selectivity of channel-pores formed by Cry46Ab mutants. Membrane currents
506 though channel-pores formed by the Cry46Ab mutants were recorded with a 4-fold gradient of KCl
507 across the lipid bilayer. The experiment was independently repeated 5 times for the K155E and
508 E159K mutants and 11 times for the E163K mutant. The average (standard deviation) V_R was
509 determined using each fitted line.
510
511 Fig. 6 Cation selectivity (K^+ vs. Na^+) of channel-pores formed by Cry46Abs. Membrane currents
512 though the channel-pores formed by Cry46Abs were recorded under asymmetric buffer conditions
513 (150 mM KCl in the *cis* chamber, 150 mM NaCl in the *trans* chamber) across the lipid bilayer. The
514 experiment was independently repeated 3 times, and the average (standard deviation) V_R was
515 determined using each fitted line.
516
517 Fig. 7 Cation selectivity (Ca^{2+} vs. K^+) of channel-pores formed by Cry46Abs. Membrane currents
518 though channel-pores formed by the Cry46Abs were recorded under asymmetric buffer conditions
519 (75 mM $CaCl_2$ in the *cis* chamber, 150 mM KCl in the *trans* chamber) across the lipid bilayer. The
520 experiment was independently repeated 3 times, and the average (standard deviation) V_R was
521 determined using each fitted line.
522
523 Fig. 8 Cation selectivity (Ca^{2+} vs. Na^+) of channel-pores formed by Cry46Abs. Membrane currents
524 though channel-pores formed by the Cry46Abs were recorded under asymmetric buffer conditions

525 (75 mM $CaCl_2$ in the *cis* chamber, 150 mM NaCl in the *trans* chamber) across the lipid bilayer. The
526 experiment was independently repeated 3 times, and the average (standard deviation) V_R was
527 determined using each fitted line.