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# Mutagenic Analysis of Putative Domain II and Surface Residues in Mosquitocidal *Bacillus thuringiensis* Cry19Aa Toxin

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Cry19Aa has high toxicity to Anopheles stephensi and Culex pipiens but is less toxic against Aedes aegypti. To study the functional role of putative domain II and surface residues, 16 alanine substitution mutations were introduced into Cry19Aa. All mutant constructs were expressed as 65-kDa protoxins in Bacillus thuringiensis 4Q7 and trypsinized, resulting in 40-kDa toxins. Among these, Y410A, W416A, D418A, F485A, W357A and Y324A mutated proteins (muteins) showed significant reduction or loss of function in mosquitocidal activities toward A. aegypti and C. pipiens. These data suggest that these residues of domain II are important in toxicity and might be involved in receptor binding.

Cry19Aa has been isolated, sequenced, and expressed from *Bacillus thuringiensis (Bt*) subsp. *jegathesan* serotype H28a28c (1, 2). Cry19Aa is highly toxic to *Culex pipiens, C. quinquefasciatus* and *Anopheles stephensi,* but has low activity against *Aedes aegypti* (3, Table 1). Cry19Aa, about 65-kDa in size, and Orf2 are co-expressed as one operon in *Bt* cells. Orf2 might be important to stabilize expression of Cry19Aa. Homolog scanning and site-directed mutagenesis techniques have been used extensively in *Bt* Cry toxins, within each domain, to localize regions responsible for toxicity, receptor binding and pore formation. For mutagenic analysis, a homology model structure of Cry19Aa was obtained based on the model structure of Cry4Ba as a template (3). Recently, the Cry4Ba structure was solved by Boonserm *et al.* (4), but we could not model Cry19Aa from the coordinates of Cry4Ba. Thus, the two sequences were aligned using Clustal X (ver. 1.83) in Fig. 1 in order to compare the loop regions of domain II. This alignment indicated that loops of the determined Cry4Ba structure were not different from our predicted Cry19Aa model. In the present study, loops of domain II and predicted surface residues in Cry19Aa were analyzed by alanine substitution mutagenesis to localize the regions and residues responsible for toxicity.

The Cry19Aa loops and surface residues mutated in this study are shown in Fig. 2. The residues Y410, E411, Y412, I413, Y414, W416, D418, V420 in loop 2, T484 and F485 in loop 3, Y356 and W357 in loop 1, Y324 and F325 in loop 0, and the two surface residues T390 and T610 were mutated to alanine. All mutant constructs were electroporated into acrystalliferous *Bt* 4Q7 strain, expressed as 65-kDa protoxins and trypsinized to 40-kDa toxins with bovine trypsin (Fig. 3 and 4). The mutated protein (mutein) F485A mutant was relatively poorly expressed compared with other mutants. Expression and trypsin digestion assay data

TABLE 1. Mosquitocidal toxicity of Cry19Aa against *Aedes* and *Culex*.

| | $LC_{50}$ (ng/ml)[a] | | |
|---|---|---|---|
| | *A. aegypti* | *C. quinquefasciatus* | *C. pipiens* |
| This study | 2.4 X 105 (1.1-6.7) | 7.3[c] (1.1-23.3) | 15.3 (9.8-28.3) |
| AEM2004[b] | 1.4 X 105 (0.4-103) | 35 (22-52) | 6 (3-9) |

[a]Two-day-old larvae were used. Mortality was recorded after 24 h except for [c](48 h). The 95% confidence limit is shown in parentheses. Bioassays used inclusion crystal proteins and spores purified from Bt transformants. [b]Data from reference 3.

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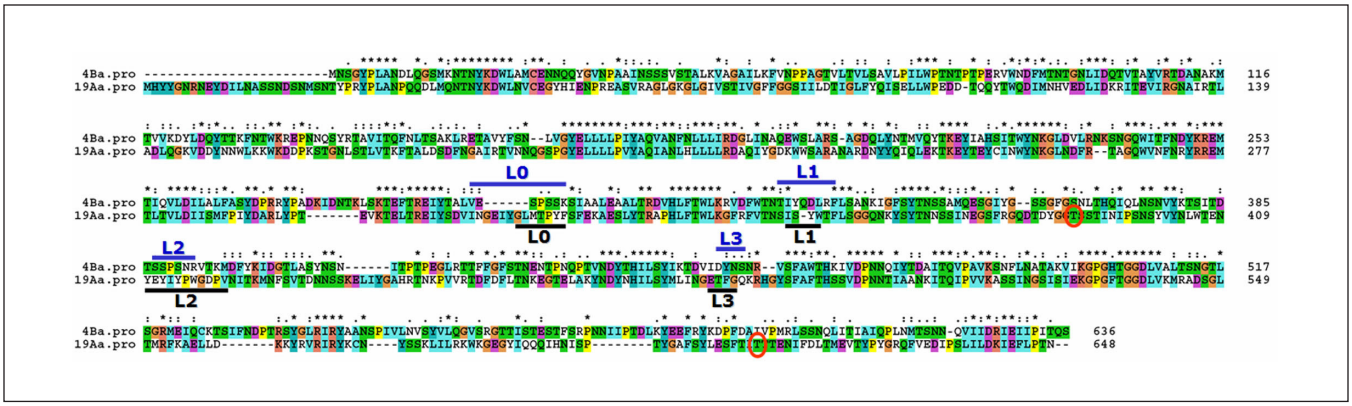



FIG. 1. Amino acid sequence alignment of Cry19Aa with Cry4Ba using by Clustal X (ver. 1.83). Upper- and underlines indicate the loops in domain II of Cry4Ba and Cry19Aa, respectively. Circled T residues are the predicted surface residues that were mutated.

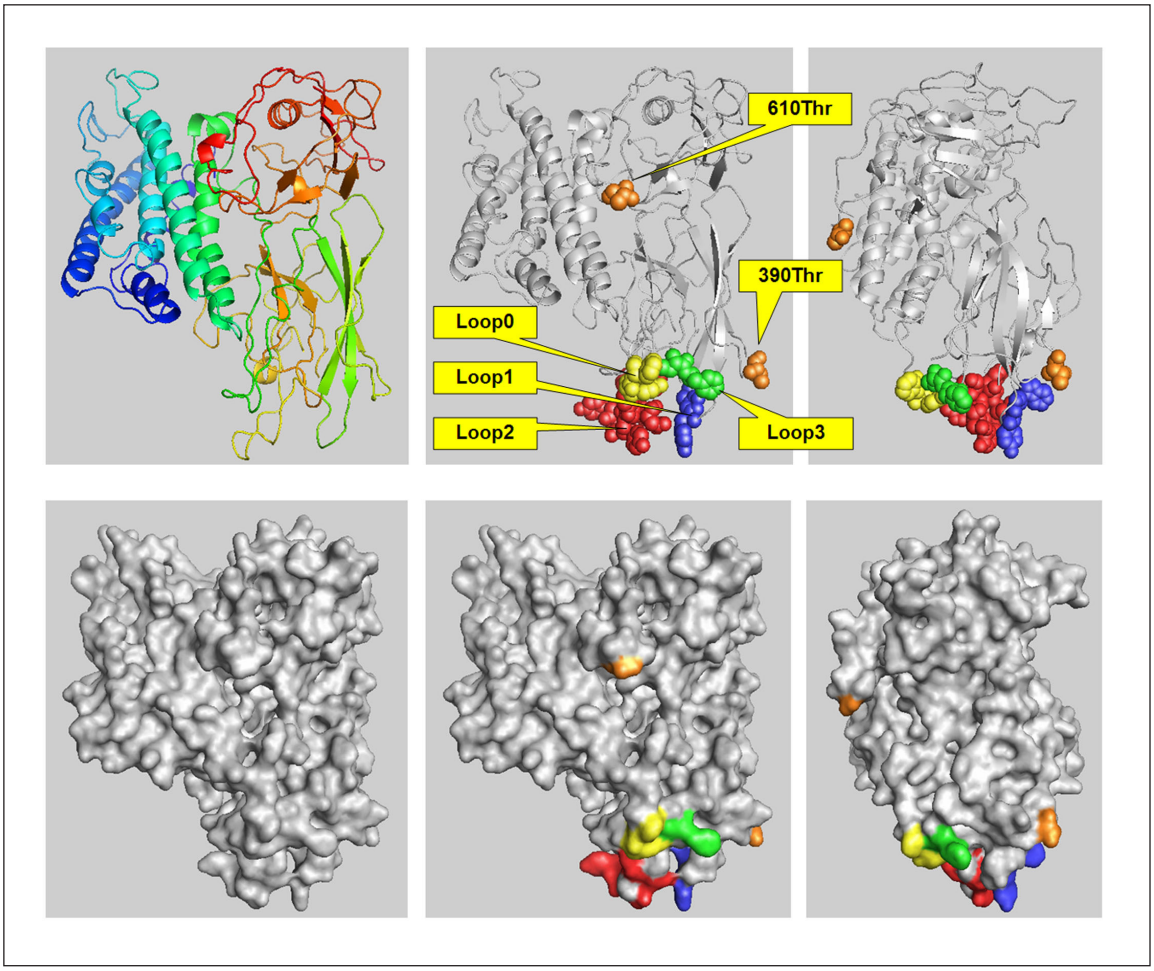



FIG. 2. Ala mutation sites on Cry19Aa model structure. The 3-D model of Cry19Aa is based on the model structure of Cry4Ba.

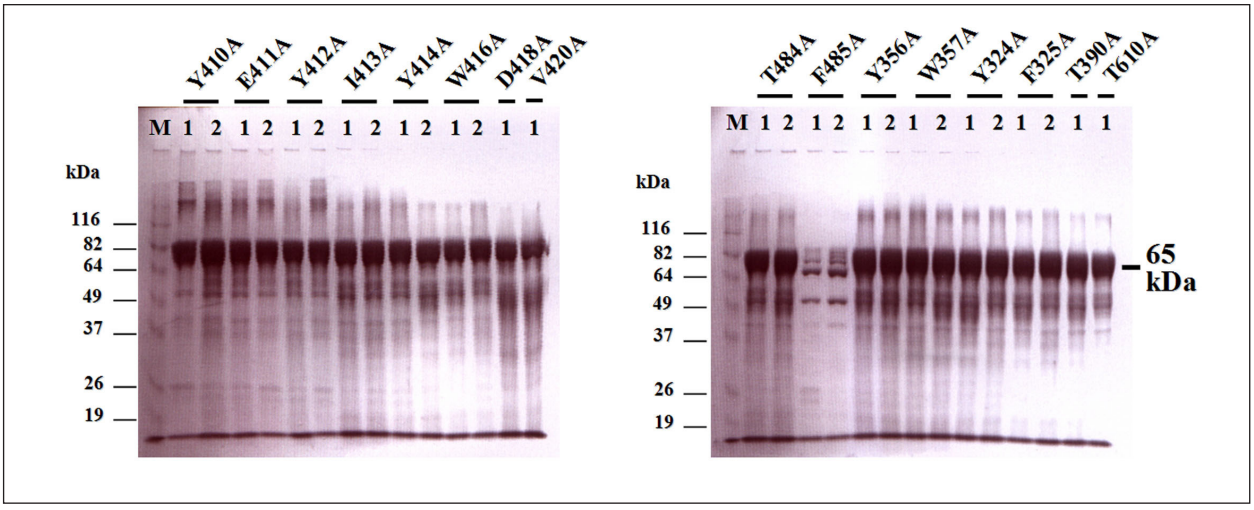



FIG. 3. Expression of Cry19Aa Ala muteins in Bt 4Q7 cells. Ala substitutions were constructed according to the Quick Change Mutagenesis method (Stratagene) using *Pfu* polymerase. Each construct was transformed into Bt 4Q7 strain. Transformants were cultured on nutrient agar plates with erythromycin (25 μ g/ml). Crystal proteins and spores were harvested after 4 days post inoculation. SDS-PAGE was performed onto 10% separating gel.

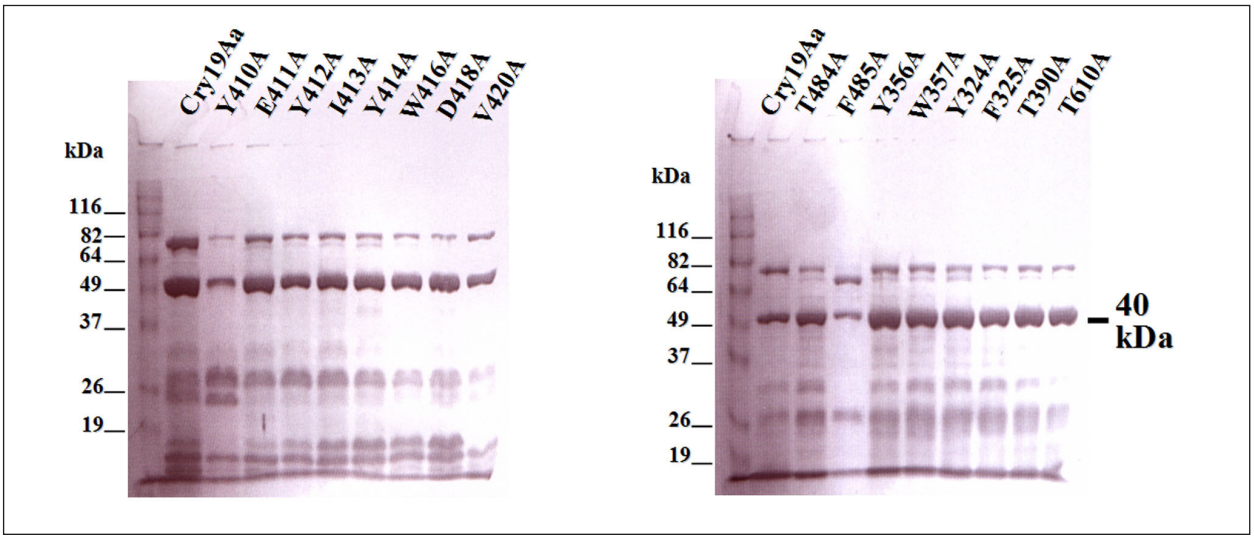
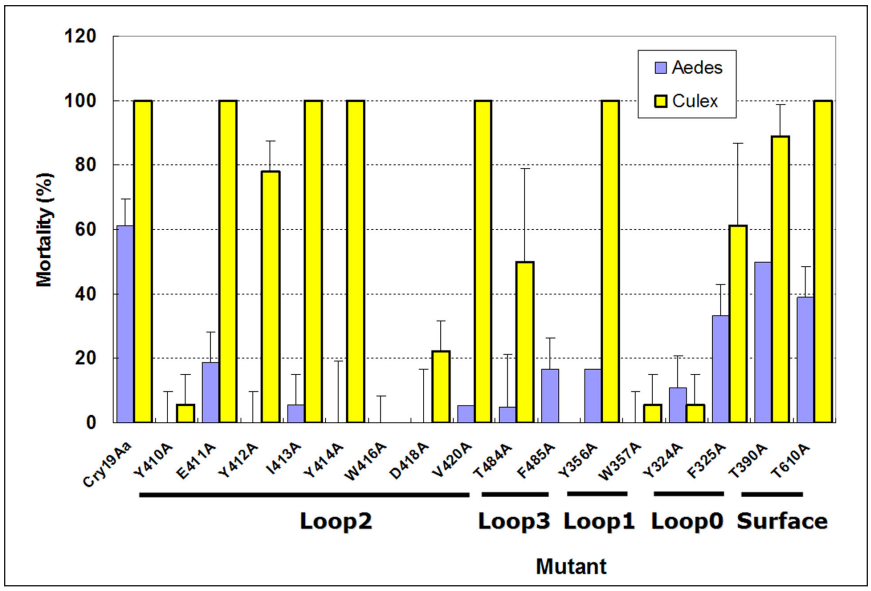



FIG. 4. Trypsin activation of Cry19Aa Ala muteins. Solubilized protoxins were adjusted to pH 7 with 2 N HCl and then treated with 20:1 (toxin: trypsin w/w) trypsin for 1 h at 37°C. SDS-PAGE was performed onto 10% separating gel.

suggested that the Cry19Aa alanine muteins might be as stable as the wild type protein. However, further experiments, such as circular dichroism assay or thermal denaturation, are needed to confirm structural stability. Mosquitocidal activity of the muteins against *A. aegypti* and *C. pipiens* was determined and compared with that of Cry19Aa (Fig. 5). All alanine substitutions in loop 1, 2 and 3 showed a large reduction in toxicity to *Aedes*. In particular, the Y410A, W416A, D418A, F485A, W357A and Y324A muteins showed significant reduction or loss of toxicity toward both *Aedes* and *Culex*. However, surface residue substitutions did not affect toxicity. These data suggest that domain II of Cry19Aa is important for toxicity as shown in the




| Mutant | | Mortality (%) | |
|---|---|---|---|
| | | *Aedes* | *Culex* |
| | Cry19Aa | +++* | +++# |
| Loop 2 | | | |
| | Y410A | - | - |
| | E411A | + | +++ |
| | Y412A | - | +++ |
| | I413A | - | +++ |
| | Y414A | - | +++ |
| | W416A | - | - |
| | D418A | - | + |
| | V420A | - | +++ |
| Loop 3 | | | |
| | T484A | - | ++ |
| | F485A | + | - |
| Loop 1 | | | |
| | Y356A | + | +++ |
| | W357A | - | - |
| Loop 0 | | | |
| | Y324A | + | - |
| | F325A | ++ | ++ |
| Surface | | | |
| | T390A | +++ | +++ |
| | T610A | ++ | +++ |

FIG. 5. Bioassay (left) and comparative toxicities (right) of Cry19Aa Ala mutants against *Aedes* and *Culex*. * Two-day-old larvae were used. The toxin concentration was 100 mg/ml for *A. aegypti* and 500 ng/ml for *C. pipiens*. Mortality was recorded after 24 h (*C. pipiens*) or 48 h (*A. aegypti*). Bioassays were performed with inclusion crystal proteins and spores purified from Bt transformants. * Comparative toxicity in *Aedes*: -, 0-10% mortality; +, 10-30%; ++, 30-50%; +++; > 50%. # Comparative toxicity in *Culex*, -, 0-10%; +, 10-40%; ++, 40-70%; +++, 70-100%.

general mode of action of Bt toxins. Further studies are needed to investigate the binding properties of Cry19Aa muteins to mosquito BBMVs.

Cry19Aa shows a higher toxicity than Cry4Aa or Cry11Aa to *C. pipiens* (5) and also has low cross resistance to *Culex* made resistant to Cry4Aa, Cry4Ba and Cry11Aa (6). These properties indicate that Cry19Aa is a valuable tool in mosquito control and resistance management.

Thus, mutagenic analysis of Cry19Aa could be used through protein engineering to enhance toxicity toward *Culex* or *Aedes* as well as to understand of the molecular mechanism of mosquitocidal toxin.

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