Highly Effective Broad Spectrum Chimeric Larvicide That Targets Vector Mosquitoes Using a Lipophilic Protein

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Two mosquitocidal bacteria, Bacillus thuringiensis subsp. israelensis (Bti) and Lysinibacillus sphaericus (Ls) are the active ingredients of commercial larvicides used widely to control vector mosquitoes. Bti’s efficacy is due to synergistic interactions among four proteins, Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa, whereas Ls’s activity is caused by Bin, a heterodimer consisting of BinA, the toxin, and BinB, a midgut-binding protein. Cyt1Aa is lipophilic and synergizes Bti Cry proteins by increasing midgut binding. We fused Bti’s Cyt1Aa to Ls’s BinA yielding a broad-spectrum chimeric protein highly mosquitocidal to important vector species including Anopheles gambiae, Culex quinquefasciatus, and Aedes aegypti, the latter an important Zika and Dengue virus vector insensitive to Ls Bin. Aside from its vector control potential, our bioassay data, in contrast to numerous other reports, provide strong evidence that BinA does not require conformational interactions with BinB or microvillar membrane lipids to bind to its intracellular target and kill mosquitoes.

Mosquitoes transmit many pathogens that cause debilitating diseases including the viruses that cause Dengue, West Nile, Zika and Yellow Fever, nematodes responsible for River Blindness and filariasis, and protozoans causing various malarias. Over half the human population lives in areas where these mosquito-vectored pathogens are endemic, with the principal vectors being species of Aedes, Anopheles, and Culex mosquitoes. Recent data from the World Health Organization show that more than 3 billion people are at risk of malaria alone, with an estimated 214 million cases and greater than 438,000 deaths in 2015, most of the latter being children who die under the age of 5, making malaria the leading cause of morbidity and mortality worldwide1. The incidence of Dengue and Yellow Fever is also high, with respectively, 50–100 million and 200,000 cases occurring yearly2, 3.

Synthetic chemical insecticides are still used to control mosquitoes. However, their detrimental environmental effects and resistance to these in target populations4 led to the development of commercial larvicides based on two mosquitocidal bacteria, Bacillus thuringiensis subsp. israelensis (Bti) and Lysinibacillus sphaericus (Ls). Both produce mosquitocidal protein crystals and have been used widely in mosquito control programs for decades5-7. When ingested, these protein crystals dissolve in the alkaline larval midgut, are proteolytically activated, and bind to microvillar receptors forming lesions that destroy midgut cells leading to larval death5-9. Cyt1Aa (27.5 kDa) differs from the Bti Cry and Ls proteins in that it does not require a glycoprotein receptor, but rather binds with high affinity to lipids in the microvillar plasmlemma10. It has only low toxicity to mosquito larvae, but is important to toxicity in that it synergizes Bti Cry and Ls mosquitocidal proteins and delays resistance to these11. After binding, Cyt1Aa is thought to act by forming pores or lipid faults in the microvillar membrane10.

The Ls binary toxin (Bin) is a heterodimer of two related propeptides, BinA, a toxin (27 kDa), and BinB, a midgut microvillar binding protein (51 kDa), which co-crystallize during synthesis5,6,12-14. BinB binds to a glycoprotein receptor, the first identified being a glycosylphosphatidylinositol (GPI)-anchored α-glucosidase15. Most Aedes and many Anopheles species lack this type of receptor and thus are not sensitive to Bin15. Unlike Bti proteins
that act at the microvillar surface, BinA and BinB are internalized and act intracellularly killing cells by autophagy and/or apoptosis\textsuperscript{5, 16, 17}, during which large cytoplasmic vacuoles are formed followed by midgut exfoliation that results in larval death. Several studies suggest that interaction of BinA and BinB is required for toxicity\textsuperscript{18–21}. At LC\textsubscript{90} levels, Ls mortality peaks at 48 hours post-treatment due to Bin's internalization process, whereas with Bti maximum mortality occurs at 24 hours post-treatment.

Results

Previous recombinant Bti strains we constructed containing various combinations of mosquitocidal Cry proteins, Cyt1Aa, and Bin are much more potent than wild type strains of Bti and Bs, and avoid resistance\textsuperscript{13, 14}. These recombinants demonstrate bacterial insecticides can be improved significantly through genetic engineering and synthetic biology techniques, suggesting other novel combinations of high efficacy are possible. In a proof-of-concept study, we fused the Cyt1Aa protoxin, which has high affinity for mosquito microvilli lipids, to the BinA protoxin, yielding the chimeric protein, Cyt1Aa-BinA (69.6 kDa). We then evaluated this construct for stable synthesis in 4Q7, an acrystalliferous strain of Bti, and for the efficacy of this recombinant chimeric strain against larvae of mosquito species belonging to the three most important genera of disease vectors, Anopheles, Aedes, and Culex. Here we show this chimeric strain forms a stable parasporal inclusion in Bti and is highly toxic against larvae of mosquito species belonging to the three most important genera of disease vectors, Anopheles, Aedes, and Culex. The high toxicity we obtained against Ae. aegypti is potentially important, as our chimera expanded the target spectrum of BinA to include this species, which lacks a BinB receptor and thus is poorly sensitive to Ls Bin\textsubscript{1}.

The Bti 4Q7 strain that synthesized Cyt1Aa-BinA (Fig. 1A) produced spores and parasporal bodies within 24–36 hr of incubation in NBG broth or on Nutrient agar (Fig. 1B). The parasporal bodies were released from fully lysed cells, or remained associated with the spore. The chimeric strain kept in NBG broth or Nutrient agar was stable for at least six months at 4°C, as determined by microscopy and SDS-PAGE. To show parasporal bodies contained the Cyt1Aa-BinA chimera, they were separated from spores on a sucrose gradient and analyzed by SDS-PAGE and Western blot analyses. A single protein of ~70 kDa, the predicted mass of Cyt1Aa-BinA, was observed, and this protein reacted with anti-Cyt1Aa and anti-BinA antibodies (respectively, Fig. 2A and B). When subjected to digestion with trypsin, the Cyt1Aa-BinA chimera yielded fragments consistent with normal cleavage products of Cyt1A and BinA (Fig. 2C).

Against all larvae, bioassays using the Cyt1Aa strain showed negligible toxicity, with LC\textsubscript{50}s ranging from 4,219–47,370 ng/ml and LC\textsubscript{95}s from 13,722–155,050 ng/ml (Table 1). Bti 4Q5, a strain that produces its four...
major toxins (Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa), was the most potent (LC$_{50}$s 3.6–7.1 ng/ml, LC$_{95}$s from 18.5–88 ng/ml). Ls 2362 was active against $C.\text{ quinquefasciatus}$, and $A.\text{ gambiae}$ and $A.\text{ stephensi}$, but not against $A.\text{ aegypti}$ and $C.\text{ quinquefasciatus}$ BS-R, a strain selected for high levels of resistance to Bin; the LC$_{50}$s and LC$_{95}$s of Ls 2362 were $>$1,000,000 ng/ml. The Cyt1Aa-BinA chimeric strain, however, was highly toxic to larvae of species belonging to all three major genera of disease vectors, $Culex$, $Aedes$ and $Anopheles$, with LC$_{50}$s ranging from 9.2 to 61.9 ng/ml, and LC$_{95}$s from 30 to 271 ng/ml (Table 1). Toxicity of the chimera was high by 24 hours post-treatment (Table 1), which typically only occurs by 48 hours when Ls is tested against larvae (Table 2).

Interestingly, with regard to both LC$_{50}$s and LC$_{95}$s, the relative toxicities of the Cyt1Aa-BinA protein (23.0 ng/ml) and Bti 4Q5 (26.5 ng/ml) against $A.\text{ gambiae}$ overlapped, those of Cyt1Aa-BinA (28.9 ng/ml) and Bti 4Q5 (14.8 ng/ml) against $A.\text{ stephensi}$ did not. However, their LC$_{95}$s completely overlapped against both species indicating that the Cyt1Aa-BinA fusion protein alone was as effective as the wild-type Bti 4Q5.

Perhaps most interesting are the LC$_{50}$s and LC$_{95}$s toxicities observed for Cyt1Aa-BinA against $A.\text{ aegypti}$, respectively, 61.9 ng/ml and 271.1 ng/ml, when compared to Ls ($>$1,000,000 ng/ml), i.e., the chimera was $>$16,155 and $>$3689 more toxic than Ls.

Preliminary histological studies of treated versus control larvae showed that the midgut epithelium was completely destroyed in moribund and dead larvae by eight hours post-treatment at the LC$_{95}$ level (Fig. 2D). Most midgut cells had sloughed from the basement membrane and had lysed. Those that still had a recognizable cellular structure lacked microvilli and had one or two large vacuoles in the cytoplasm, the characteristic cytopathology resulting from Ls Bin intoxication.

In the present study we fused the protoxins, not the activated toxins, so that the protoxin chimera contained proteolytic cleavage sites of each partner. Once activated in the midgut lumen each partner should then act independently, Cyt1Aa causing midgut microvillar membrane lesions through which BinA would enter the cytoplasm to reach its internal target site, killing the cell within 24 hr rather than the 48 hours required by the BinAB complex$^{5,22,23}$. Our trypsin activation and Western blot results (Fig. 2C) indicate the two partners separated...
### Table 1. Toxicity of the Cyt1Aa-BinA chimera and control strains of *Bacillus thuringiensis* subsp. *israelensis* (Bti) and *Lysinibacillus sphaericus* (Ls) to 4th-instar larvae of *Anopheles* mosquitoes.

| Bacterial Strain (Toxins produced) | LC$_{50}$ (Fiducial Limits) | RT$^4$-LC$_{50}$ | LC$_{95}$ (Fiducial Limits) | RT$^4$-LC$_{95}$ | Slope |
|------------------------------------|-----------------------------|------------------|-----------------------------|------------------|-------|
| *Culex quinquefasciatus* S-Lab     |                             |                  |                             |                  |       |
| Bti 4Q7/pWF45 (Cyt1Aa)             | 47,370.1 (35,629.5–59,705.4) | 6.671            | 155,050.3 (114,040.8–260,168.4) | 1,914.2           | 3.2 ± 0.5 |
| Ls 2362 (BinAB)                    | 181.4 (118.1–296.3)         | 25.6             | 7,569.6 (3,009.6–33,192.5)    | 93.5             | 1.0 ± 0.1 |
| Bti 4Q7/cyt1Aa-binA (Cyt1Aa-BinA chimera) | 9.2 (7.2–12.1)               | 1.3              | 30.5 (20.2–71.2)              | 0.4              | 3.2 ± 0.6 |
| Bti 4Q5 (Cry4A, Cry4B, Cry11A, Cry1A) | 7.1 (2.6–18.6)               | 1.0              | 81.0 (16.7–427.3)             | 1.0              | 1.6 ± 0.4 |

**Culex quinquefasciatus Bti**$^5$

| Bti 4Q7/pWF45 (Cyt1Aa)             | 27,022.4 (20,523.2–35,262.4) | 5.630            | 100,175.2 (70,685.6–169,448.0) | 4,595.2           | 2.9 ± 0.4 |
| Ls 2362 (BinAB)                    | >1,000,000$^5$               | >208,333         | >1,000,000$^5$                | >45,872           | —      |
| Bti 4Q7/cyt1Aa-binA (Cyt1Aa-BinA chimera) | 10.2 (2.6–41.4)              | 2.1              | 42.0 (2.2–1,214.9)            | 1.9              | 2.7 ± 1.0 |
| Bti 4Q5 (Cry4A, Cry4B, Cry11A, Cry1A) | 4.8 (3.5–6.3)                | 1.0              | 21.8 (14.4–45.3)              | 1.0              | 2.5 ± 0.4 |

**Anopheles gambiae**

| Bti 4Q7/pWF45 (Cyt1Aa)             | 4,218.7 (2,997.1–5,709.8)    | 1.172            | 22,764.9 (14,634.9–48,334.1)  | 84               | 2.3 ± 0.4 |
| Ls 2362 (BinAB)                    | >1,000,000                   | >277,778         | >1,000,000                    | >3,688           | —      |
| Bti 4Q7/cyt1Aa-binA (Cyt1Aa-BinA chimera) | 61.9 (46.7–80.1)             | 17.2             | 271.1 (185.1–513.4)           | 14.7             | 2.6 ± 0.4 |
| Bti 4Q5 (Cry4A, Cry4B, Cry11A, Cry1A) | 3.6 (2.5–4.8)                | 1.0              | 18.5 (11.9–39.6)              | 1.0              | 2.3 ± 0.4 |

**Anopheles stephensi**

| Bti 4Q7/pWF45 (Cyt1Aa)             | 46,557.3 (13,980.9–137,613.6) | 1.757            | 129,978.8 (25,520.6–923,387.1) | 1,465.4           | 3.7 ± 1.4 |
| Ls 2362 (BinAB)                    | 201.2 (154.6–260.4)          | 7.6              | 1,150.9 (760.7–2,206.5)       | 13               | 2.2 ± 0.3 |
| Bti 4Q7/cyt1Aa-binA (Cyt1Aa-BinA chimera) | 23.0 (17.6–30.0)             | 0.9              | 80.9 (57.4–135.4)             | 0.9              | 3.0 ± 0.4 |
| Bti 4Q5 (Cry4A, Cry4B, Cry11A, Cry1A) | 26.5 (20.2–34.3)             | 1.0              | 88.7 (63.9–144.8)             | 1.0              | 3.1 ± 0.4 |

And acted independently, achieving toxicity within 24 hr for all mosquito species and strains tested (Table 1) as opposed to 48 hr with wild type BinAB. Our purpose did not include determining the type of Cyt1Aa lesion formed. However, with a diameter of about 3 nm, BinA is too large to be a cation ion channel (1–2 nm) and more likely forms an irregular lipid fault as opposed to a larger semicircular pore. In fact, in a previous study we showed that the BinAB complex, about 6 nm in diameter, can enter Cx. quinquefasciatus midgut cells resistant to Bin in vivo through Cyt1Aa lesions without binding to microvilli.

### Discussion

High toxicity to *Ae. aegypti* (Table 1) was unexpected because this species does not have a Bin receptor and Cyt1Aa’s effect is negligible. However, we previously showed combination of Ls technical powder with purified Cyt1Aa crystals at a 1:1 ratio increased toxicity slightly to *Ae. aegypti*, with LC$_{50}$ and LC$_{95}$ values of 3,800 ng/ml and 31,500 ng/ml, respectively. With a diameter of about 3 nm, BinA is too large to be a cation ion channel (1–2 nm) and more likely forms an irregular lipid fault as opposed to a larger semicircular pore. In fact, in a previous study we showed that the BinAB complex, about 6 nm in diameter, can enter Cx. quinquefasciatus midgut cells resistant to Bin in vivo through Cyt1Aa lesions without binding to microvilli.

Aside from potential vector control applications, the Cyt1Aa-BinA chimera could prove useful for clarifying how BinA kills midgut cells causing mosquito death. The literature on these topics is full of disparate and often contradictory results. Although not as toxic to *Ae. aegypti* as Bti 4Q5 with the wild type parasporal body (LC$_{50}$ = 3.6 ng/ml, LC$_{95}$ = 18.5 ng/ml), these results demonstrate that the Cyt1Aa-BinA chimera strain extended the target spectrum of Ls BinA (Table 1). Thus, rather than using a mixture of Bti and Ls, as is currently done in some current commercial products, the Cyt1Aa-BinA chimera combines the properties of high toxicity against a broad vector target spectrum with the known resistance management properties of Cyt1Aa.

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independent dissociation or tetramer formation is lacking in any of these studies. In earlier studies, it was shown expression of the bin operon, i.e., binA and binB, yielded only a single crystal, demonstrating BinA and BinB formed a heterodimer, not separate crystals, which was confirmed recently by the solution of Bin’s crystal structure. Another problem with a report that BinA and BinB prepared separately and then mixed together, BinB formed a heterodimer, not separate crystals, which was confirmed recently by the solution of Bin’s crystals. We do not question these results under the conditions tested, but our in vivo results reported here provide strong evidence that BinA once activated is highly toxic without requiring BinB for conformational changes, nor does it appear to require interactions with microvillar membrane lipids for toxicity. This suggests that BinA’s hydrophobic domains may target this toxin to an intracellular organelle, such as the endoplasmic reticulum, rather than act by forming pores in the microvillar membrane.

Materials and Methods

Bacterial strains, culture media, and DNA extraction. The DH5α strain of Escherichia coli (Invitrogen) was used for cloning and amplifying plasmid DNA. The strains of crystalliferous B. thuringiensis subsps. israelensis (Bti) 4Q5, acryl crystalliferous Bti 4Q7, and L. sphaericus (Ls) 2363 were obtained from the Bacillus Genetic Stock Center (Ohio State University, Columbus, OH). Erythromycin-resistant recombinants 4Q7/pWF45 and 4Q7/p45S1, producing, respectively, Cyt1Aa and BinA/BinB (42 kDa/51 kDa) parasporal bodies have been described previously. All strains were maintained on Nutrient agar (Becton Dickinson, Sparks, MD) throughout the study. LB medium (Becton Dickinson, Sparks, MD) was used for growing E. coli and extracting plasmid DNA using the Wizard Plus Mini-prep DNA Purification system (Promega). Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen).

Construction of pBU-cyt1Aa-binA. To make a construct that synthesizes the Cyt1Aa-BinA chimera, plasmid pWF53 was digested with Sall and HindIII (FastDigest, Thermo Scientific) and the 1.4-kb fragment that contains the cyt1Ac promoter controlling expression of the 20-kDa chaperone-like gene was ligated into plasmid pBU2 digested with the same enzymes and treated with FastAP alkaline phosphatase (Thermo Scientific) to contain the chaperone-like gene was ligated into plasmid cry1Ac 20-kDa cry1Ac operon, i.e., binA and binB, yielded only a single crystal, demonstrating BinA and BinB formed a heterodimer, not separate crystals, which was confirmed recently by the solution of Bin’s crystal structure. Another problem with a report that BinA and BinB prepared separately and then mixed together, BinB formed a heterodimer, not separate crystals, which was confirmed recently by the solution of Bin’s crystals. We do not question these results under the conditions tested, but our in vivo results reported here provide strong evidence that BinA once activated is highly toxic without requiring BinB for conformational changes, nor does it appear to require interactions with microvillar membrane lipids for toxicity. This suggests that BinA’s hydrophobic domains may target this toxin to an intracellular organelle, such as the endoplasmic reticulum, rather than act by forming pores in the microvillar membrane.

Table 2. Toxicity of the Cyt1Aa-BinA chimeric strain of Bacillus thuringiensis subsps. israelensis 4Q7/Cyt1Aa-BinA or Lysinibacillus sphaericus (Ls) wild type strain to 4th-instars of Culex quinquefasciatus at 24 versus 48 hours post treatment. *ng/ml.

| Bacterial Strain | LC50 (Fiducial Limits)* | LC95 (Fiducial Limits)* | Slope |
|------------------|------------------------|------------------------|-------|
| 24 hours         |                        |                        |       |
| Ls 2362 (BinAB)  | 218.7 (158.5–308.0)    | 2,548.5 (1,396.8–6,667.3) | 1.5 ± 0.2 |
| Bti 4Q7/cyt1Aa-binA (Cyt1Aa-BinA chimera) | 6.5 (3.3–13.0) | 38.5 (11.0–174.6) | 2.1 ± 0.5 |
| 48 hours         |                        |                        |       |
| Ls 2362 (BinAB)  | 19.5 (14.7–25.9)       | 90.5 (61.5–160.3)      | 2.5 ± 0.3 |
| Bti 4Q7/cyt1Aa-binA (Cyt1Aa-BinA chimera) | 5.8 (2.7–12.5) | 31.0 (7.6–151.8) | 2.3 ± 0.6 |

Transformation. Bti 4Q7 was transformed by electroporation as previously described, and transformants (4Q7/pBU-cyt1Aa-binA) were selected on LB agar with tetracycline (3 μg/ml) at 28°C.

Bacterial strains and purification of parasporal bodies. Ls 2362 was grown in MBS broth, and Bti strains 4Q5, 4Q7/pWF45, 4Q7/pBU-cyt1Aa-binA, and 4Q7/p45S1 were grown in 50 ml of NBG appropriately supplemented with 25 μg/ml erythromycin and 3 μg/ml tetracycline, at 28°C for 4 days by which time >95% of the cells had sporulated and lysed. Spores and crystals were collected by centrifugation at 6,500 g for 15 min, washed 2x in double-distilled (dd) H2O, followed by centrifugation at 6,500 g for 15 min at 4°C after each wash, and lyophilized (FreezeZone 4.5, Labconco) for storage.

To isolate parasporal bodies, spore/parasporal body mixtures collected from 50 ml cultures were resuspended in 15 ml ddH2O and sonicated twice at 50% duty cycle for 15 s using the Ultrasonic Homogenizer 4710 (Cole-Parmer Instrument Co.). Five-milliliter samples were loaded onto a sucrose gradient cushion (30–65% w/v), which was then centrifuged at 20,000 g for 45 min at 20°C in a Beckman L7–55 ultracentrifuge using the
Western blot analysis. Purified parasporal bodies (~10 µg) were solubilized in alkaline buffer (50 mM Na2CO3, pH 11) and protein concentration was determined by the method of Bradford, as described previously13,14. Protein samples (0.75 µg and 1.5 µg) were fractionated by electrophoresis in an SDS–10% polyacrylamide gel and electrophoretically onto a polyvinylidene difluoride membrane (MicronSeparations, Inc.) using a model PSS50 electroblotter (Hoefer Scientific Instruments). Western blot analysis was performed using primary rabbit anti-BinA and anti-Cyt1A antibodies and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Southern Biotechnology Associates, Inc., Birmingham, AL) as the secondary antibody. Binding of the secondary antibody was detected with the nitroblue tetrazolium and 5-bromo-1-chloro-3-indolyl phosphate (BCIP) reagents (Promega).

Tryptsin digest. Approximately 5 µg of purified parasporal bodies were solubilized in 25 µl 50 mM NaOH, 25°C for 10 min, followed by addition of 25 µl of 50 mM HCl. Samples were spun at 16,000 g for 5 min to remove the insoluble fraction, and supernatants were collected and activated with 1:50 (w/w) trypsin (Sigma) for 2 h at 25°C. The products liberated by proteolytic cleavage were analyzed by SDS-PAGE as previously described13,14.

Microscopy. Sporulating cultures were monitored and photographed with a DMRE phase-contrast microscope (Leica) at a magnification of 1,000x. For preliminary histological studies, control, moribund, and dead larvae (LC50 level) were fixed, dehydrated, and embedded in Epon-Araldite14. Sections 0.25–0.50 µm thick were cut and examined with the above phase contrast microscope.

Bioassays. Lyophilized cultures containing spores and parasporal bodies of the Bti and Ls strains were resuspended in ddH2O. Suspensions were diluted to 6 to 7 different concentrations, ranging from 0.5 ng/ml to 1 µg/ml, in 6 oz cups in a final volume of 100 ml. Bioassays were replicated three times using 30 fourth-instars of S-Lab (Bin-sensitive) and BS-R (Bin-resistant) strains of Cx. quinquefasciatus. Ae. aegypti, An. gambiae (courtesy of B. J. White, Department of Entomology, University of California, Riverside, CA) and An. stephensi (courtesy of A. A. James, Department of Molecular Biology and Biochemistry, University of California, Irvine) per concentration. After a 24 h exposure at 28°C, dead larvae were counted and the 50% and 95% lethal concentrations, respectively, LC50 and LC95, were calculated by Probit analysis (POLO-PC; LeOra Software, Berkeley, CA)14.

Availability of data. All reagents and data described in this manuscript are available upon request.

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Author Contributions
D.K.B., H.-W.P. created the Cyt1Aa-BinA fusion, and constructed the plasmids for synthesis in Bacillus
thuringiensis. B.A.F. and R.H.H. contributed to the concept and assisted with the experiments. The bioassays were
carried out by M.C.W. and H.-W.P. and they did the statistical analyses. D.K.B., H.-W.P. and B.F. interpreted the
results and wrote the paper with assistance from R.H. and M.C.W.

Additional Information
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