RECOMBINANT LARVICIDAL BACTERIA WITH MARKEDLY IMPROVED EFFICACY AGAINST CULEX VECTORS OF WEST NILE VIRUS

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Abstract. An urgent need exists for new agents to control mosquito vectors of disease. Mosquito larvicides based on the bacteria Bacillus thuringiensis subsp. israelensis (Bti) or B. sphaericus (Bs) are effective in many habitats, but use is limited by their high cost. Moreover, mosquito resistance evolves rapidly to Bs where it is used intensively. The efficacy of these bacteria is due to a binary protein (BsB) in Bs and four proteins (Cry4A, Cry4B, Cry11A, and Cyt1A) in Bti. Here we report the use of cyt1A promoters and a 5’ mRNA stabilizing sequence to synthesize high levels of Bs2362 binary toxin in Bti strains. The recombinant BtiIPS-82/BsB showed high potency against fourth instars of Culex quinquefasciatus, a vector of West Nile virus, being 21-fold as potent as BtiIPS-82, and 32-fold as potent as Bs2362. Similar improved efficacy was obtained against larvae of Cx. tarsalis. Moreover, BtiIPS-82/BsB suppressed resistance to Bs2362 in Cx. quinquefasciatus.

INTRODUCTION

Despite advances in medical science, mosquito-borne diseases including malaria, filariasis, dengue, and the viral encephalitides remain the most important diseases of humans, with an estimated two billion people worldwide living in areas where these are endemic.1 Thus, there is an urgent need for new agents and strategies to control these diseases. Potential strategies include vaccines, new drugs, and transgenic mosquitoes refractory to the causative disease agents, but in the near future control efforts will rely on insecticides and existing drugs.

Since World War II, disease control methods have relied heavily on broad spectrum synthetic chemical insecticides to reduce vector populations. However, chemical insecticides are being phased out in many countries due to insecticide resistance in mosquito populations. Furthermore, many governments restrict chemical insecticide use due to concerns over their environmental effects on non-target beneficial insects, and especially on vertebrates through contamination of food and water supplies. As a result, the World Health Organization is facilitating the replacement of these chemicals with bacterial insecticides through the development of standards for their registration and use.2

Vector control products based on bacteria are designed to control larvae. The most widely used are VectoBac® and Teknar®, which are based on Bacillus thuringiensis subsp. israelensis (Bti). In addition, VectoLex®, a product based on B. sphaericus (Bs), has come to market recently for control of mosquito vectors of filariasis and viral diseases. All three of these products are manufactured by Valent BioSciences (Libertyville, IL). These products have achieved moderate commercial success in developed countries, but their high cost deters use in many developing countries. Moreover, concerns have been raised about their long-term use due to resistance, which has already been reported to B. sphaericus in field populations of Culex mosquitoes in Brazil, China, France, and India (Singère G and others, unpublished data).3–5

The insecticidal properties of these bacteria are due primarily to insecticidal proteins produced during sporulation. In Bti, the key proteins are Cyt1A (27 kD), Cry11A (72 kD), Cry4A (128 kD), and Cry4B (134 kD), whereas Bs produces 41.9-kD (BinA) and 51.4-kD (BinB) proteins that serve as the toxin and binding domains of a single binary toxin, respectively.6,7 In previous studies, we and others have shown that Cyt1A synergizes the toxicity of Cry4A, Cry4B, and Cry11A and delays resistance to these compounds.8–11 Moreover, we have also shown that Cyt1A can suppress resistance to Bs strain 2362 and expand its target spectrum.12,13

The biochemical and toxicologic differences between the Bti and Bs toxins and the novel toxicologic properties of Cyt1A suggested that it might be possible to construct improved mosquitocidal bacteria by recombining their toxins using recombinant DNA technology. Several recombinants have been constructed since the late 1980s, but none had toxicity sufficiently better than wild type Bti or Bs to warrant commercial development.14–20 Here we report the application of recent molecular genetic techniques we developed for enhancing endotoxin synthesis21,22 to construct novel recombinant bacteria that synthesize high levels of the Bs2362 binary toxin in acrystalliferous and crystalliferous Bti strains. The recombinant strain that produces a combination of Bti and Bs toxins is at least 20-fold more toxic than either of the parental strains to larvae of Culex quinquefasciatus and Cx. tarsalis. Aside from its high efficacy, this new bacterium is much less likely to induce resistance in target populations because it combines Cyt1A with Bti Cry toxins and the Bs binary toxin. The markedly improved efficacy and resistance-delaying properties of this new bacterium make it an excellent candidate for development and use in vector control programs, especially to control Culex vectors of West Nile virus and other viruses, as well as species of this genus that transmit filarial diseases.

MATERIALS AND METHODS

Bacterial strains, genes, plasmids, and transformation. The B. sphaericus strain 2362 was provided by Valent BioSciences. The Escherichia coli–B. thuringiensis shuttle expression vector pHT310123 was used to construct and amplify plasmid pPHSP-1 in E. coli DH5α. The pPHSP-1 construct was expressed in the acrystalliferous 4Q7 strain of B. thuringiensis subsp. israelensis (obtained from the Bacillus Genetic Stock Center at Ohio State University, Columbus, OH) or in B. thuringiensis subsp. israelensis IPS-82 (Institut Pasteur, Paris, France). The modified pHT3101-based vector (pSTAB) con-
taining the 660-basepair fragment with the cytIA promoters and STAB-SD sequence has been previously described.21,22 Plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). The Bacillus strains were transformed by electroporation.22

**Amplification of the B. sphaericus binary toxin operon by a polymerase chain reaction (PCR).** A crude plasmid preparation was made from B. sphaericus 2362 by alkaline lysis. The gene encoding the 51.4-kD and 41.9-kD binary toxins of B. sphaericus (GenBank M20390) was obtained by PCR using Vent (Exo+) DNA polymerase (New England Biolabs, Beverly, MA) and the primers BSP-1 (5'-aactgcagCTTGT-CAACATGTGAAGATTAAAAGGTAACTTTCAG-3') and BSP-2 (5'-aactgcagCCAAACAACACAGTTTACATTC-GAGTGTAAGGTTC-3'). The underlined sequences in the primers are Pst I sites. The 3.4-kb PCR product was digested with Pst I and cloned into the Pst I site in pHST3101 to generate pHBS (Figure 1A). The 3.0-kb Hpa I-Pst I fragment in pHBS was cloned into the filled Xba I and Pst I sites in pSTAB (Figure 1B) to generate plasmid pPHSP-1 (Figure 1C) for production of the Bs2362 binary toxin in the Bacillus strains.

**Growth of bacterial strains.** The strains B. thuringiensis subsp. israelensis 4Q7/pPHSP-1 (Bti4Q7/BsB) and B. thuringiensis subsp. israelensis IPS-82/pPHSP-1 (BtiIPS-82/BsB) were grown on nutrient agar (BBL Microbiology Systems, Cockeysville, MD) or in peptonized milk (1% peptonized milk [BBL Microbiology Systems], Cockeysville, MD) or in peptonized milk (1% peptonized milk [BBL Microbiology Systems], 1% dextrose, 0.2% yeast extract, 1.216 mM MgSO4, 0.072 mM FeSO4, 0.139 mM ZnSO4, 0.118 mM MnSO4) with erythromycin (Fisher Scientific, Pittsburgh, PA) at a concentration of 25 μg/mL. Bacillus sphaericus strains were grown in MBS medium.25 For insect bioassays, BtiIPS-82/BsB was grown in 25 mL of peptonized milk with erythromycin (25 μg/mL) in a shaker incubator (250 rpm) for 5 days at 28°C during which time > 98% of the cells had sporulated and lysed. Spores and crystals were harvested by centrifugation at 6,000 × g for 15 minutes at 4°C. The pellet was washed twice in water and dried in a vacuum chamber.

**Quantification of endotoxin yields per unit medium.** After growth in peptonized milk, 1 mL of each lysed culture was collected and centrifuged at 10,000 × g for 5 minutes. The supernatant was discarded and 150 μL of 2 × sample buffer was added. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).26 To quantify the levels of Bs2362 toxin, a standard curve was established using different quantities of purified binary toxin and scanning densitometry.

**Mosquito strains.** Two strains of Cx. pipiens quinquefasciatus (referred to as Cx. quinquefasciatus) were used: BS-R, a strain resistant to B. sphaericus 2362, and S-Lab, an selected, sensitive strain. Strain BS-R has been selected with since 1992 and routinely survives 48 hours of exposure to 1,000 μg/mL of Bs2362 technical powder, a concentration 149,000-fold higher than the concentration that kills 50% of S-Lab, the sensitive reference strain. The sensitive S-Lab strain of Cx. quinquefasciatus was established from mosquitoes collected in California.27 This colony has been maintained in the laboratory without exposure to B. sphaericus. Other mosquito species tested included Cx. tarsalis, a colony established from larvae field-collected in 2000 in southern California, a laboratory colony of Aedes aegypti,13 and a laboratory colony of Anopheles albimanus (obtained from the United States Department of Agriculture, Agricultural Research Laboratory, Gainesville, FL).

**Selection for resistance and bioassay procedures.** The BS-R strain of Cx. quinquefasciatus was selected for resistance to Bs2362 by exposing groups of approximately 1,000 early fourth-instar larvae to 250–400 μg/mL of technical powder in enameled metal pans in approximately 1 liter of deionized water for 48–96 hours. Average larval mortality under selection was ≤ 10% per selection, and the survivors were used to continue the colony. For bioassays, groups of 20 early fourth instars were exposed to a range of concentrations of the lyophilized spore/crystal powders of wild-type and recombinant Bacillus strains in 100 mL of deionized water held in 237-mL plastic cups. Seven to nine different concentrations of the powders, which yielded mortality between 2% and 98% after 48 hours, were replicated on three different days. All data were subjected to probit analysis using a program for the PC.28 Dose-response values with overlapping confidence intervals were not considered to be significantly different.

**Microscopy.** Sporulating cultures were monitored by light microscopy with a Zeiss (Thornwood, NY) Photomicroscope III using a 100 × oil-immersion objective. For transmission electron microscopy, sporulated cells from peptonized milk cultures were collected just before lysis, fixed for 2 hours in 3% cacodylate-buffered glutaraldehyde and 0.25% sucrose, post-fixed in 1% OsO4, dehydrated in ethanol-propylene oxide, and embedded in Epon-Araldit.29 Ultrathin sections of sporulated cells were stained with uranyl acetate and lead citrate, examined and photographed in a Hitachi (Pleasanton,

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**Figure 1.** Schematic illustration of the cloning and engineering of the Bacillus sphaericus (Bs) strain 2362 binary toxin operon. The operon (A) was cloned using a polymerase chain reaction, placed under the control of the cytIA-pSTAB-SD expression system (B), and cloned (C) into the Escherichia coli-B. thuringiensis shuttle vector pHST3101 to yield plasmid pHSP-1 that produced high levels of the Bs binary toxin in B. thuringiensis subsp. israelensis after transformation. E.coli ori = E. coli origin of replication; Amp = ampicillin; Em = erythromycin; bp = basepairs.
CA) 600 electron microscope operating at an accelerating voltage of 75 kV. For scanning electron microscopy, purified Bs2362 crystals were plated onto a stub, air-dried, coated with 60% gold/40% palladium, and observed using a Phillips (Sunnyvale, CA) XL30 scanning electron microscope. Crystal dimensions were measured on scanning electron micrographs. The length and width of 10 crystals were measured for each strain. Data were analyzed for statistical significance using the Super Analysis of Variance (ANOVA) program (Abacus Concepts, Berkeley, CA).

**Quantification of vegetative cell and spore yields.** One milliliter of *B. thuringiensis* culture for each strain was collected at two points: 1) one hour before sporulation initiation to estimate the number of vegetative cells, and 2) after cell lysis to estimate the number of spores. For the spore count, each culture was incubated at 60°C for 20 minutes to inactivate vegetative cells. After serial dilution, cultures were plated on nutrient agar plates supplemented with 25 μg/ml of erythromycin, and incubated for 20 hours at 30°C to determine number of vegetative cells and spores produced per milliliter.

Data were analyzed with the Super ANOVA program (Abacus Concepts). The experiments were repeated three times on three different days with three different cultures.

**RESULTS**

**Bs2362 binary toxin synthesis in Bti strains.** Synthesis of the Bs2362 binary toxin in acrystalliferous Bti strain (4Q7) harboring the recombinant plasmid pPHSP-1 (Figure 1), referred to hereafter as strain Bti4Q7/BsB, typically resulted in the production of one large toxin crystal per cell outside the exosporium membrane (Figure 2A), making the crystals easy to purify (Figure 2B). In wild-type Bs, the toxin crystal is produced inside the exosporium membrane that also surrounds the spore, keeping the crystal associated with the spore. The crystal has an unusual shape with many facets. Thus, for the purpose of measurements, the crystal was considered a rectangular block with equal sides serving as the short axis and the long axis being the length. For measurements on scanning electron micrographs, mean dimensions for Bs crystals produced in Bti4Q7/BsB were 0.80 μm × 0.80 μm wide by 1.0 μm long, yielding a volume of 0.64 μm³. Crystals produced in wild type Bs2362 averaged 0.42 μm × 0.42 μm wide by 0.58 μm long, yielding a volume of 0.10 μm³. This resulted in an approximate size increase of more than six-fold for crystals produced using the cyt1A-p/STAB-SD expression system in BtiIPS-82/BsB compared with wild-type Bs2362 crystals. Wild-type crystals proved difficult to purify because of their comparatively small size and enclosure within the exosporium membrane. Thus, most measurements used to calculate a size increase had to be made on transmission electron micrographs. The median length of crystals produced by Bti4Q7/BsB was 1.0 μm, as determined by scanning electron microscopy (Figure 2B).

When pPHSP-1 was used to synthesize the Bs Bin protein in BtiIPS-82 along with the normal complement of Bti proteins, this strain, referred to hereafter as BtiIPS-82/BsB, yielded BsB crystals (Figure 2C) that averaged 0.77 μm × 0.77 μm width by 1.0 μm length, yielding a volume of 0.59 μm³. This was not statistically different compared with the size of crystals produced by Bti4Q7/BsB.

With respect to stability, our initial results indicate that the recombinant strains are stable. Bti4Q7/BsB and BtiIPS-82/BsB have each been cultured for more than 50 successive generations using various growth media in the presence or absence of selection pressure. Over this period, we have not observed any plasmid loss or decrease in endotoxin production.

**Analysis of endotoxin synthesis by SDS-PAGE.** Synthesis of the Bs2362 toxin in Bti4Q7/BsB and BtiIPS-82/BsB demonstrated a substantial increase in toxin yield per unit medium as assessed by scanning densitometry (Figure 2D). The wild-type Bs2362 strain produced approximately 100 μg/mL of the 41.9-kD and 51.4-kD binary toxin proteins (Figure 2D, lane 1), whereas the yield of these proteins was 223 μg/mL (Figure 2D, lane 2) in Bti4Q7/BsB and 302 μg/mL in BtiIPS-82/BsB (Figure 2D, lane 3). The higher level of binary toxin production per unit medium by BtiIPS-82/BsB than that by Bti4Q7/BsB (Figure 2D) was likely due to higher level of sporulation obtained with the former strain (Table 1). The total yield of Bti Cry and Cyt1A proteins and Bs2362 binary toxin in the BtiIPS-82/BsB recombinant was 472 μg/mL, compared with approximately 250 μg/mL of Cry and Cyt1A in wild-type Bti (IPS-82), giving an increase of approximately 190% in total toxin yield per milliliter compared with Bti, and slightly more than 450% compared with wild-type Bs2362. The net endotoxin increase in BtiIPS-82/BsB resulted in a concomitant decrease of approximately 32% of the BtiIPS-82 endotoxins compared with the yield of these obtained with...
wild-type Bti (Figure 2D, lane 4 versus lane 3). Interestingly and importantly, spore yields per unit medium of the recombinant strains were significantly lower than those of wild-type strains, whereas there was no statistical difference in vegetative cell numbers between recombinants and wild types (Table 1). This indicates that the marked increase in toxicity production in the recombinant reduced sporulation.

Toxicity of bacterial strains to sensitive mosquitoes. The recombinant strains Bti4Q7/BsB and BtiIPS-82/BsB were much more toxic than either of BtiIPS-82 or Bs2362 to fourth instars of Cx. quinquefasciatus (Table 2). For example, the BtiIPS-82/BsB strain (50% lethal concentration [LC₅₀] = 0.37 ng/mL) was more than 21-fold as toxic as wild-type BtiIPS-82 (LC₅₀ = 8.1 ng/mL) at 24 hours post-treatment. Compared with toxicity of wild-type Bs2362 at 48 hours post-treatment, the time at which Bs assays are typically assessed due to the slower action of its binary toxin in comparison to Bti toxins, the recombinant BtiIPS-82/BsB was approximately 32-fold as toxic as Bs2362 (LC₅₀ = 11.9 ng/mL). The recombinant Bti4Q7/BsB strain (LC₅₀ = 1.4 ng/mL) that only produced the Bs2362 toxin was only approximately eight-fold as toxic as wild-type Bs2362 (LC₅₀ = 11.9 ng/mL).

Toxicity improvements were also observed for the BtiIPS-82/BsB recombinant against Cx. tarsalis (Table 2). Compared with BtiIPS-82, toxicity was 2–3-fold higher at the LC₅₀ and LC₉₅ after 24 hours and 6–10-fold higher after 48 hours. Improvement relative to Bs2362 was 8–20-fold after 24 hours and 4–6-fold after 48 hours.

With respect to Ae. aegypti and An. albimanus, the improvement in toxicity of the BtiIPS-82/BsB recombinant was not nearly as substantial as that obtained against Cx. quinquefasciatus. Against each species, the increase in activity of BtiIPS-82/BsB in comparison to either wild-type BtiIPS-82 or Bs2362 was on average 1.8-fold (Table 2).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Toxins synthesized</th>
<th>Vegetative cells × 10³/mL</th>
<th>Spores × 10³/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bti4Q7</td>
<td>None</td>
<td>14.1 (0.9) a</td>
<td>15.9 (1.9) c</td>
</tr>
<tr>
<td>BtiIPS-82</td>
<td>Cry4A, Cry4B, Cry11A, Cyt1A</td>
<td>9.9 (0.9) b</td>
<td>24.9 (3.2) c</td>
</tr>
<tr>
<td>Bti4Q7/BsB</td>
<td>Bs Bin</td>
<td>19.3 (5.5) a</td>
<td>2.6 (3.0) d</td>
</tr>
<tr>
<td>BtiIPS-82/BsB</td>
<td>Cry4A, Cry4B, Cry11A, Cyt1A, Bs Bin</td>
<td>12.6 (3.1) a, b</td>
<td>5.5 (2.2) d</td>
</tr>
</tbody>
</table>

* Values followed by different letters are significantly different at P = 0.05.

Table 1
Mosquitocidal toxicity of wild-type Bacillus thuringiensis subsp. israelensis (Bti) (IPS-82) and B. sphaericus (Bs) (262) in comparison to Bti10-fold higher after 48 hours. Improvement relative to Bs2362 was 8–20-fold after 24 hours and 4–6-fold after 48 hours.

With respect to Ae. aegypti and An. albimanus, the improvement in toxicity of the BtiIPS-82/BsB recombinant was not nearly as substantial as that obtained against Cx. quinquefasciatus. Against each species, the increase in activity of BtiIPS-82/BsB in comparison to either wild-type BtiIPS-82 or Bs2362 was on average 1.8-fold (Table 2).

Table 2
Mosquitocidal toxicity of wild-type Bacillus thuringiensis subsp. israelensis (Bti) (IPS-82) and B. sphaericus (Bs) (262) in comparison with recombinant B. thuringiensis subsp. israelensis strains producing the B. sphaericus 262 binary toxin (BsB)*

<table>
<thead>
<tr>
<th>Mosquito species Strain</th>
<th>Exposure period (hours)</th>
<th>Bacterial strain†</th>
<th>LC₅₀ (confidence intervals) (ng/mL)</th>
<th>LC₉₅ (confidence intervals) (ng/mL)</th>
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<tbody>
<tr>
<td>Culex quinquefasciatus</td>
<td>S-Lab</td>
<td>24</td>
<td>BtiIPS-82</td>
<td>8.1 (6.6–10.2)</td>
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<tr>
<td></td>
<td>48</td>
<td></td>
<td>3.2 (1.8–5.9)</td>
<td>13.6 (4.1–47.7)</td>
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<tr>
<td></td>
<td>S-Lab</td>
<td>24</td>
<td>BtiIPS-82/BsB</td>
<td>0.37 (0.2–0.46)</td>
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<tr>
<td></td>
<td>48</td>
<td></td>
<td>0.014 (0.01–0.09)</td>
<td>1.8 (0.04–393)</td>
</tr>
<tr>
<td></td>
<td>S-Lab</td>
<td>24</td>
<td>Bs2362</td>
<td>499.0 (129–2,330)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>11.9 (9.4–14.7)</td>
<td>95.9 (68.4–152)</td>
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<tr>
<td>Resistant</td>
<td>BS-R</td>
<td>24</td>
<td>BtiIPS-82</td>
<td>30.1 (18.9–47.0)</td>
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<td></td>
<td>48</td>
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<td>21.6 (9.81–47.3)</td>
<td>69.4 (15.3–319)</td>
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<td>BtiIPS-82/BsB</td>
<td>10.8 (3.63–32.9)</td>
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<td></td>
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<td>9.2 (3.36–255)</td>
<td>57.2 (3.62–1,060)</td>
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<tr>
<td></td>
<td>BS-R</td>
<td>24</td>
<td>Bs2362</td>
<td>&gt; 10⁶ §</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td></td>
<td>&gt; 10⁶§</td>
<td>&gt; 10⁶§</td>
</tr>
<tr>
<td>Culex tarsalis</td>
<td>24</td>
<td>BtiIPS-82</td>
<td>59.7 (38.3–93.0)</td>
<td>364 (160–839)</td>
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<td>37.7 (25.7–55.1)</td>
<td>165 (87.6–322)</td>
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<td>24</td>
<td>BtiIPS-82/BsB</td>
<td>25.6 (19.1–34.3)</td>
<td>110 (65.2–193)</td>
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<td>48</td>
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<td>3.8 (3.2–4.6)</td>
<td>26 (19.3–39.2)</td>
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<tr>
<td></td>
<td>24</td>
<td>Bs2362</td>
<td>223 (135–370)</td>
<td>2,260 (719–7,250)</td>
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<tr>
<td></td>
<td>48</td>
<td></td>
<td>24.6 (21.5–28.0)</td>
<td>107 (86.8–138)</td>
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<tr>
<td>Aedes aegypti</td>
<td>24</td>
<td>BtiIPS-82</td>
<td>16.2 (14.2–19.3)</td>
<td>42.2 (31.7–69.5)</td>
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<tr>
<td></td>
<td>48</td>
<td></td>
<td>8.9 (6.3–21.8)</td>
<td>20.7 (3.86–116)</td>
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<tr>
<td></td>
<td>24</td>
<td>BtiIPS-82/BsB</td>
<td>6.4 (0.92–4.46)</td>
<td>31.7 (0.30–3,490)</td>
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<tr>
<td>Anopheles albimanus</td>
<td>24</td>
<td>BtiIPS-82</td>
<td>18.9 (16.2–22.2)</td>
<td>80.3 (61.8–114)</td>
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<td>48</td>
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<td>7.79 (0.12–4.54)</td>
<td>20.6 (0.01–10,700)</td>
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<tr>
<td></td>
<td>24</td>
<td>BtiIPS-82/BsB</td>
<td>7.32 (6.2–8.5)</td>
<td>27.2 (21.2–38.7)</td>
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<td>48</td>
<td></td>
<td>4.2 (3.6–5.0)</td>
<td>15.2 (12.0–21.3)</td>
</tr>
</tbody>
</table>

* LC₅₀ = lethal concentration.
† BtiIPS-82 is the wild-type BtiIPS-82 strain of B. thuringiensis subsp. israelensis. Bs2362 is the wild-type 2362 strain of B. sphaericus, and BtiIPS-82/BsB is the recombinant strain of B. thuringiensis subsp. israelensis engineered to synthesize the B. sphaericus 262 binary toxin under the control of cyt1A promoters and the STAB-SD mRNA stabilizing sequence.
‡ No mortality at 1 mg/mL.
§ 17% mortality at 1 mg/mL.
Toxicity of bacterial strains to resistant mosquitoes. The rapid emergence of resistance to Bs2362 where it has been used intensively in the field (Singère G and others, unpublished data) has raised serious concerns about the long-term viability of this strain for vector control. However, the results we obtained with the BtiIPS-82/BsB recombinant show that it suppresses extremely high levels of resistance to Bs2362, restoring sensitivity to levels comparable those obtained with BtiIPS-82 and Bs2362 against non-selected mosquitoes (Table 2). For example, whereas no mortality was obtained at 24 hours post-treatment, and only 17% at 48 hours with wild-type Bs2362 used at a rate of 1 mg/mL (>100,000-fold resistance), the LC50 was reduced to 9.2 ng/mL at 48 hours for the BtiIPS-82/BsB recombinant. This is comparable to LC50 values of 11.9 ng/mL for Bs2362 and 8.1 ng/mL for BtiIPS-82 at 48-hours and 24-hours post-treatment, respectively, against the sensitive S-Laboratory strain of Cx. quinquefasciatus.

DISCUSSION

The mosquitocidal properties of Bti and Bs differ considerably. Bti, for example, is highly toxic to a wide range of mosquito species and has a broad dipteran target spectrum beyond these, being toxic to the larvae of biting and non-biting midges, blackflies, and craneflies. In contrast, Bs is toxic only to a limited range of mosquitoes, mostly Culex species and some anophelines, produces toxins unrelated to the Cry and Cyt toxins of Bti, and persists longer than the latter species. The unique properties of these two different bacteria, along with the availability of recombinant DNA techniques, stimulated attempts since the late 1980s to combine these properties into a single bacterium more effective than Bti or Bs. Most attempts, which typically involved cloning one or more Bti genes into Bs, yielded recombinants that produced the expected endotoxin combinations, but these strains showed little if any improvement over wild-type strains, and thus did not warrant commercial development. Introduction of cry4B into Bs2362, for example, extended its target spectrum to Ae. aegypti, but the recombinant was no more effective than Bti. Similar results were obtained when cry4B and cry11A were introduced into Bs2297. The Bs/Bti recombinant had increased activity against Ae. aegypti, but was no better than wild-type Bti. Introduction of the Bs binary toxin into Bti yielded similar results. Introduction of the Bs1593 binary toxin operon into Bti yielded a strain that produced a significant quantity of Bs binary toxin. However, the recombinant was no better than the parental Bs or Bti against Ae. aegypti, Cx. pipiens, or An. stephensi. More recently, cyt1A was combined with the BsC3-41 binary toxin in acrystalliferous BtiQ7 with moderately encouraging results. This Bti/Bs recombinant was almost four-fold more toxic to Cx. pipiens quinquefasciatus than Bti1897, or a Bti strain that only produced BsC3-41 toxin. In addition, the recombinant suppressed approximately 80% of the resistance in a Cx. pipiens quinquefasciatus population highly resistant to BsC3-41 (LC90 > 10 mg/mL). Nevertheless, the toxicity of this strain was poor (LC50 = 1.120 ng/mL) compared with BtiIPS-82 (LC50 = 8-10 ng/mL) against Culex and Aedes species.

In contrast to these earlier attempts to develop a much more effective bacterium than Bti or Bs, the BtiIPS-82/BsB recombinant reported here was 21-fold more toxic than Bti-IPS-82, and 32-fold more toxic than Bs2362 to Cx. quinquefasciatus (Table 2). Similar improvement was noted for another important vector species, Cx. tarsalis. Moreover, this recombinant completely suppressed extremely high levels of resistance to Bs2362 (Table 2). However, only a low level of improvement (1.8-fold) was obtained against Ae. aegypti and An. albimanus. Nevertheless, it remains possible that further testing will show that Bti/BsB is effective against a greater range of medically important species of Aedes, Anopheles, and other genera.

The markedly improved efficacy of the BtiIPS-82/BsB recombinant results from combination of Bti proteins with a large quantity of the highly potent Bs2362 binary toxin (Figure 2). The combined level of Bs binary toxin and Bti toxins synthesized per cell was much greater than that obtained in either of the parental strains (Figure 2), or per unit of culture medium (Table 2). The significant reduction in spores produced per unit of culture medium also contributed to the much higher efficacy of this strain compared with parental strains (Table 1). This phenomenon is possibly related to cannibalism of vegetative cells at the onset of sporulation, such as that observed in sporulating B. subtilis cells. In this species, cells that have entered the sporulation pathway produce a killing factor and a signal protein that block sister cells from sporulating. This increased toxicity per unit weight resulted, in essence, from substituting endotoxin protein for about 80% of the normal weight due to spores, which are not toxic. Lastly, it is possible that synergistic interactions between the Bs2362 toxin and Bti proteins occurred and contributed to the extraordinary increase in toxicity of BtiIPS-82/BsB over the parental Bs and Bti strains. With respect to resistance, the ability of BtiIPS-82/BsB to suppress high levels of resistance to Bs2362 in Cx. quinquefasciatus is likely due to the presence of Bti proteins, especially Cyt1A, which restores the toxicity of the Bs binary protein.

Aside from its much higher efficacy than wild-type bacteria, the BtiIPS-82/BsB recombinant has other properties that make it an almost ideal larvicide. It is well documented that it is more difficult for insects to develop resistance to mixtures of toxins rather than individual toxins. The high levels of resistance already reported to Bs in the field (Singère G and others, unpublished data) compared with the apparent absence of resistance to BtiIPS-82, and 32-fold more toxic than Bs2362 to Cx. quinquefasciatus (Table 2). Similar improvement was noted for another important vector species, Cx. tarsalis. Moreover, this recombinant completely suppressed extremely high levels of resistance to Bs2362 (Table 2). However, only a low level of improvement (1.8-fold) was obtained against Ae. aegypti and An. albimanus. Nevertheless, it remains possible that further testing will show that Bti/BsB is effective against a greater range of medically important species of Aedes, Anopheles, and other genera.

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Our results suggest that the new BtiIPS-82/BsB recombinant will be very useful for control of vector mosquitoes, especially Culex and certain Anopheles species. Although the potential species that could be controlled worldwide are too numerous to mention, a few examples of species controlled with B. sphaericus are worth noting. These are Cx. quinque-
fasciatus, Cx. tarsalis, Cx. nigripalpus, and Culex pipiens in the United States, An. darlingi and An. aquasalis in the Amazon basin in Brazil, and An. gambiae and sibling species in Africa. These anopheline species are known to be sensitive to B. sphaericus, and this bacterium is used to control An. darlingi and An. aquasalis, important malaria vectors, in Brazil. The Culex species noted are the primary vectors of West Nile virus in the United States. Many Culex species also serve as the vectors of the filarial worms that cause filariasis in southeast Asia. Moreover, although we have emphasized the use of BitIPS-82/ BsB for vector mosquito control, its high efficacy may also make it suitable for use against other dipteran vectors and nuisance biting flies throughout many regions of the world. These would include nuisance mosquitoes, midges, and blackflies, as well as important blackfly vectors, such as Simulium damnosum, a vector of Onchocerca volvulus, a filarial worm that causes onchocerciasis, a severely debilitating eye disease of humans in west Africa.

In summary, we have used recombinant DNA technology to develop a remarkably improved novel insecticidal bacterium, which is much more toxic than available commercial strains, and with built-in resistance management properties. This new bacterium could prove useful for controlling a variety of vector and nuisance flies in many regions of the world.

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