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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 synthesis[21,22] 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 pHT3101[23] 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) containing the 660-basepair fragment with the *cyt1A* promoters and STAB-SD sequence[24] 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′-aactgcagCTTGTCAACATGTGAAGATTAAAGGTAACTTTCAG-3′) and BSP-2 (5′-aactgcagCCAAACAACAACAGTTTACATTCGAGTGTAAAAGTTC-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 pHT3101 to generate pHBS (Figure 1A). The 3.0-kb *Hpa* I-*Pst* I fragment in pHBS was cloned into the filled *Xb*a 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], 1% dextrose, 0.2% yeast extract, 1.216 mM $MgSO_4$, 0.072 mM $FeSO_4$, 0.139 mM

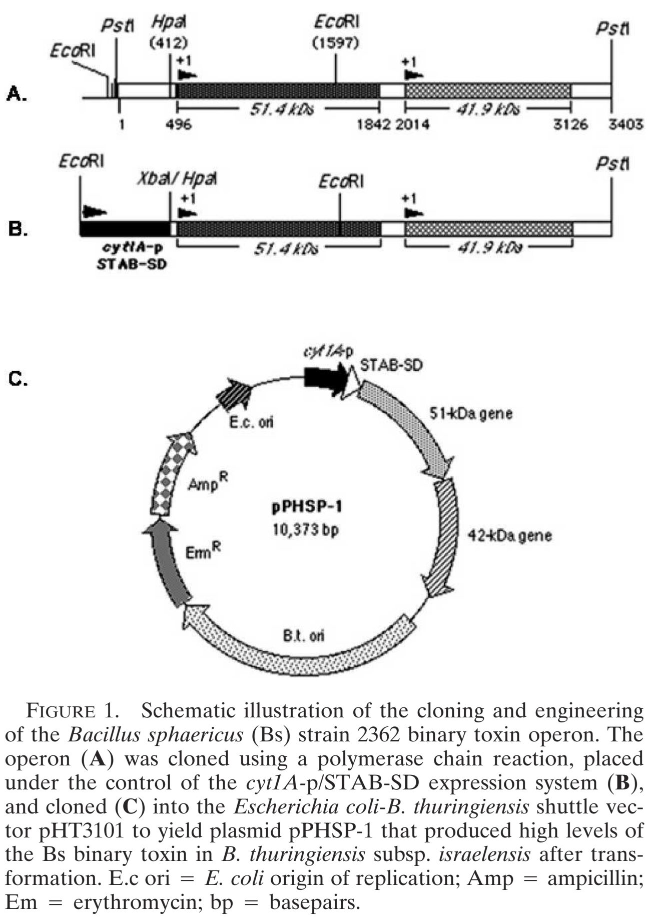

FIGURE 1. Schematic illustration of the cloning and engineering of the *Bacillus sphaericus* (Bs) strain 2362 binary toxin operon. The operon (**A**) was cloned using a polymerase chain reaction, placed under the control of the *cyt1A*-p/STAB-SD expression system (**B**), and cloned (**C**) into the *Escherichia coli-B. thuringiensis* shuttle vector pHT3101 to yield plasmid pPHSP-1 that produced high levels of the Bs binary toxin in *B. thuringiensis* subsp. *israelensis* after transformation. E.c ori = *E. coli* origin of replication; Amp = ampicillin; Em = erythromycin; bp = basepairs.

$ZnSO_4$, 0.118 mM $MnSO_4$) 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[26] 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 unselected, sensitive strain. Strain BS-R has been selected with since 1992[12] 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-instars to 100–120 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[28] using a program for the PC.[29] 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% OsO_4, dehydrated in ethanol-propylene oxide, and embedded in Epon-Araldite.[9] Ultrathin sections of sporulated cells were stained with uranyl acetate and lead citrate, examined and photographed in a Hitachi (Pleasanton, 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[8] 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 was 0.80 μm × 0.80 μm wide by 1.0 μm long, yielding a volume of 0.64 μm^3. 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 μm^3. This resulted in an approximate size increase of more than six-fold for crystals produced using the *cyt1A*-p/STAB-SD expression system in Bti4Q7/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 μm^3. This was not statistically different compared with the size of crystals produced by Bti4Q7/BsB.

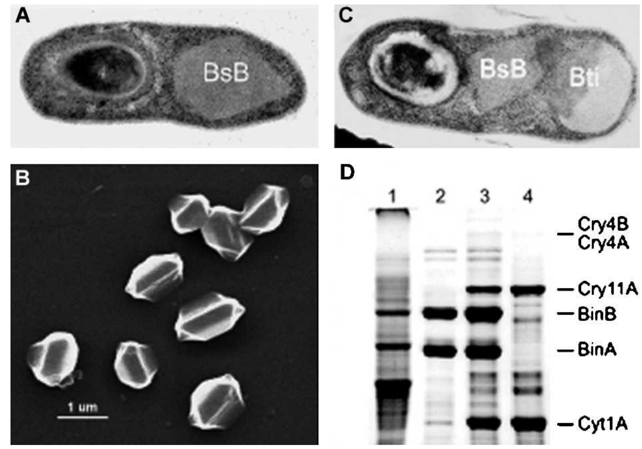

FIGURE 2. Synthesis and purification of *Bacillus sphaericus* 2362 binary toxin in *B. thuringiensis* subsp. *israelensis*. **A**, Transmission electron micrograph of the recombinant acrystalliferous strain Bti4Q7/BsB, which was engineered to synthesize the *B. sphaericus* binary toxin. The single large crystal (**BsB**) is adjacent to the spore. **B**, Scanning electron micrograph of purified *B. sphaericus* 2362 binary toxin crystals produced in the recombinant strain Bti4Q7/BsB. Bar = 1.0 μm. **C**, Transmission electron micrograph of the recombinant crystalliferous strain BtiIPS-82/BsB, which was engineered to synthesize the *B. sphaericus* binary toxin. This recombinant strain produces the typical IPS-82 parasporal body (**Bti**) and Bs2362 binary toxin crystal (**BsB**). **D**, Comparative endotoxin yields produced per unit medium by wild-type *B. sphaericus* strain 2362 and wild-type and engineered strains of *B. thuringiensis* subsp. *israelensis* constructed to synthesize the Bs2362 binary toxin. Lane 1, wild-type control *B. sphaericus* 2362 strain; lane 2, recombinant Bti4Q7 strain Bti4Q7/BsB, which was engineered to produce Bs2362 toxin; lane 3, recombinant BtiIPS-82 strain BtiIPS-82/BsB that produces the Bs2362 toxin and the typical Bti parasporal body; lane 4, wild-type control Bti IPS-82 strain.

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

TABLE 1

Number (SD) of vegetative cells prior to sporulation in comparison to spore yields post-sporulation for wild-type and recombinant strains of *Bacillus thuringiensis* subsp. *israelensis* (Bti) that produce the *B. sphaericus* (Bs) 2362 binary toxin (BsB)*

Bacterial strain	Toxins synthesized	Vegetative cells $\times 10^7$/mL	Spores $\times 10^7$/mL
Bti4Q7	None	14.1 (0.9) a	15.9 (1.9) c
BtiIPS-82	Cry4A, Cry4B, Cry11A, Cyt1A	9.9 (0.9) b	24.9 (3.2) c
Bti4Q7/BsB	Bs Bin	19.3 (5.5) a	2.6 (3.0) d
BtiIPS-82/BsB	Cry4A, Cry4B, Cry11A, Cyt1A, Bs Bin	12.6 (3.1) a, b	5.5 (2.2) d

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

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 toxin 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 [LC_{50}] = 0.37 ng/mL) was more than 21-fold as toxic as wild-type BtiIPS-82 (LC_{50} = 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 (LC_{50} = 11.9 ng/mL). The recombinant Bti4Q7/BsB strain (LC_{50} = 1.4 ng/mL) that only produced the Bs2362 toxin was only approximately eight-fold as toxic as wild-type Bs2362 (LC_{50} = 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 LC_{50} and LC_{95} 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 2

Mosquitocidal toxicity of wild-type *Bacillus thuringiensis* subsp. *israelensis* (Bti) (IPS-82) and *B. sphaericus* (Bs) (2362) in comparison with recombinant *B. thuringiensis* subsp. *israelensis* strains producing the *B. sphaericus* 2362 binary toxin (BsB)*

Mosquito species	Strain	Exposure period (hours)	Bacterial strain†	LC_{50} (confidence intervals) (ng/mL)	LC_{95} (confidence intervals) (ng/mL)
Culex quinquefasciatus					
Sensitive	S-Lab	24	BtiIPS-82	8.1 (6.65–10.2)	61.4 (38.6–126)
		48		3.2 (1.8–5.9)	13.6 (4.1–47.7)
	S-Lab	24	BtiIPS-82/BsB	0.37 (0.29–0.46)	5.5 (3.6–10.1)
		48		0.014 (0.01–0.9)	1.8 (0.04–393)
	S-Lab	24	Bs2362	499.0 (129–2,330)	6,350 (149–54,718)
		48		11.9 (9.4–14.7)	95.9 (68.4–152)
Resistant	BS-R	24	BtiIPS-82	30.1 (18.9–47.0)	104.0 (43.9–255)
		48		21.6 (9.81–47.3)	69.4 (15.3–319)
	BS-R	24	BtiIPS-82/BsB	10.8 (3.63–32.9)	72.0 (3.25–1,910)
		48		9.2 (3.36–255)	57.2 (3.62–1,060)
	BS-R	24	Bs2362	$> 10^6$‡	$> 10^6$
		48		$> 10^6$§	$> 10^6$
Culex tarsalis		24	BtiIPS-82	59.7 (38.3–93.0)	364 (160–839)
		48		37.7 (25.7–55.1)	165 (87.6–322)
		24	BtiIPS-82/BsB	25.6 (19.1–34.3)	110 (65.2–193)
		48		3.8 (3.2–4.6)	26 (19.3–39.2)
		24	Bs2362	223 (135–370)	2,260 (719–7,250)
		48		24.6 (21.5–28.0)	107 (86.8–138)
Aedes aegypti		24	BtiIPS-82	16.2 (14.2–19.3)	42.2 (31.7–69.5)
		48		8.9 (3.63–21.8)	20.7 (3.86–116)
		24	BtiIPS-82/BsB	6.4 (0.92–44.6)	31.7 (0.30–3,490)
		48		4.8 (4.14–5.59)	14.1 (11.1–20.5)
Anopheles albimanus		24	BtiIPS-82	18.9 (16.2–22.2)	80.3 (61.8–114)
		48		7.79 (0.12–454)	20.6 (0.01–10,700)
		24	BtiIPS-82/BsB	7.32 (6.2–8.5)	27.2 (21.2–38.7)
		48		4.2 (3.6–5.0)	15.2 (12.0–21.3)

* LC = lethal concentration.
† BtiIPS-82 is the wild-type IPS-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* 2362 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)[3–5] 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 LC_{50} was reduced to 9.2 ng/mL at 48 hours for the BtiIPS-82/BsB recombinant. This is comparable to LC_{50} 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[30] 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.[7,31] The unique properties of these two different bacteria, along with the availability of recombinant DNA techniques, stimulated attempts since the late 1980s to recombine these properties into a single bacterium more effective than Bti or Bs.[14–20] 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.[14] 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.[16] 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.[17] 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 Bti4Q7 with moderately encouraging results.[20] 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 (LC_{50} > 10 mg/mL). Nevertheless, the toxicity of this strain was poor (LC_{50} = 1,120 ng/mL) compared with BtiIPS-82 (LC_{50} = 8-10 ng/mL) against *Culex* and *Aedes* species.[6,9,10]

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 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.

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.[32,33] 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.[34] 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.[12]

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.[35–37] The high levels of resistance already reported to Bs in the field (Singère G and others, unpublished data).[3–5] compared with the apparent absence of resistance to Bti[38] show that this principle applies to bacterial larvicides. The BtiIPS-82/BsB recombinant contains five major toxins in comparison to four in Bti and the single binary toxin of Bs (Table 1). Equally if not more important is the presence of the Cyt1A protein in combination with the Bti Cry proteins and the Bs binary toxin. It is known that Cyt1A can suppress resistance to Bti Cry proteins[34] and the Bs binary toxin,[11] and there is strong evidence that it is more important to delaying resistance than the presence of a low number of multiple related toxins.[10] Thus the BtiIPS-82/BsB recombinant is not only much more effective than either parental strain, but has much better properties for delaying or avoiding mosquito resistance because it brings together Cyt1A with a greater number of toxins.

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 BtiIPS-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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