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Protection from UV-B Damage of Mosquito Larvicidal Toxins from *Bacillus thuringiensis* subsp. *israelensis* Expressed in *Anabaena* PCC 7120

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Abstract. A transgenic strain of the nitrogen-fixing filamentous cyanobacterium *Anabaena* PCC 7120 protected expressed δ-endotoxin proteins of *Bacillus thuringiensis* subsp. *israelensis* from damage inflicted by UV-B, a sunlight component that penetrates Earth's ozone layer. This organism, which serves as a food source to mosquito larvae and could multiply in their breeding sites, may solve the environment-imposed limitations of *B. thuringiensis* subsp. *israelensis* as a mosquito biological control agent.

Various subspecies of *Bacillus thuringiensis* (*Bt*) are distinct by producing during sporulation large quantities of insect larvicidal proteins (δ-endotoxins) aggregated in parasporal crystalline bodies [28]. The high potencies and specificities of these insecticidal crystal proteins (ICP) have spurred their use as natural pest control agents in agriculture, forestry, and human health [28]. Subsp. *israelensis* has widely been used as an efficient and safe biological agent to control larvae of mosquitoes and black flies, vectors of many human infectious diseases and a nuisance in temperate zones [20]. The mosquito larvicidal properties of *Bt* subsp. *israelensis* (*Bti*) are attributed to synergistic interactions among six proteins in the crystals—Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, Cyt1Aa, and Cyt2Ba [8, 20, 23]—the genes of which are located on the 137-kb plasmid pBtoxis [4]. The primary target of the ICPs is the alkaline midgut epithelium, where proteolytic enzymes cleave the solubilized pro-toxin to the active respective polypeptides that disrupt membrane permeability [16, 17]. The midgut cells consequently lyse, and the organism dies. Application of *Bti* for mosquito control is limited by short residual activity of current preparations under field conditions [20].

A major problem affecting the efficacy and economy of *Bt* is its inactivation by sunlight in the field [2, 14, 15, 22, 24, 25]. The UV-A/B range (280–380 nm) of sunlight reaching Earth's surface is considered responsible for substantial photo-degradation and consequent loss of toxicity [24, 25]. Different formulations have been developed with additional synthetic screens [7, 9, 21], but the major drawback is their negative impact on soil and water pollution. Toxicity of *Bt* has been protected from UV irradiation by natural melanin [18, 27], probably acting as a free radical trap. Among the various in vivo mechanisms counteracting UV damage is accumulation of UV-A/B-absorbing compounds [1, 6, 10, 11, 13, 29]. Various cyanobacterial pigments act as UV-B photoprotectors [11, 13]; these microorganisms are, therefore, likely to preserve the toxicity of sunlight-sensitive Cry proteins. The nitrogen-fixing filamentous cyanobacterium *Anabaena* can multiply in breeding sites of mosquito larvae and serve as their food source [30].

A clone of *Anabaena* PCC 7120 has been constructed to express a cluster of genes (on pSBJ2) for two *Bti* toxins (*cry4Aa* and *cry11Aa*) and an accessory protein (*p20*) [3] under control of two tandem strong promoters, a cyanobacterial constitutive (P_{psbA}) and an *Escherichia coli*-inducible T7 early promoter (P_{A1}) [32]. High toxicity against *Aedes aegypti* larvae was obtained at concentration of 5×10^5 cells ml^{-1}—the highest ever

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reached for an engineered cyanobacterium [32]. This clone was exploited to test the hypothesis that the transgenic *Anabaena* protects the expressed heterologous toxin proteins from the damage inflicted by UV-B (280–330 nm).

Materials and Methods

Bacterial strains. *Bti* was isolated from a primary powder (Bactimos 1990, fun 89CO6D, Duphar B.V., Weesp, Holland). *Anabaena* PCC 7120 is a laboratory-adapted strain [31] harboring pSBJ2 with *Bti* toxic gene cluster [32].

Media. Luria-Bertani (LB) (0.5% yeast extract, 1% tryptone, and 1% NaCl) used for *Bti*, and BG11 for *Anabaena* PCC 7120 [26]. Media composition included (final concentration in mM): 17.65 $NaNO_3$; 0.18 K_2HPO_4; 0.3 $MgSO_4$; 0.25 $CaCl_2$; 0.19 Na_2CO_3; 0.003 $Na_2MgEDTA$; 0.029 citric acid; and 0.03 ferric ammonium citrate, pH 8, as well as A5 trace minerals (μM): 46 B (H_3BO_3); 0.17 Co [$Co(NO_3)_2$]; 0.32 Cu ($CuSO_4$); 9.2 Mn ($MnCl_2$); 1.6 Mo (Na_2MoO_4) and 0.77 Zn ($ZnSO_4$).

Growth conditions and measurements. A single colony of *Bti* was inoculated into a tube containing 5 ml LB and incubated overnight at 30°C on a rotary shaker (200 rpm). 0.1 ml of the culture ($3–4 \times 10^8$ cells ml^{-1}) was transferred to 100 ml LB. Spores were harvested after 4 days' growth at 30°C, when the culture completed 100% sporulation and crystallization (observed by phase microscopy) and reached 1.6×10^9 spores ml^{-1}.

Transgenic *Anabaena* PCC 7120 was grown at 30°C in BG11 medium with neomycin (25 μg ml^{-1}) in an incubator-rotary shaker (160 rpm) with irradiance of 50 μmol photon. m^{-2} s^{-1} (continuous illumination). The cells were washed thrice with sterile distilled water before each experiment to remove traces of nutrients. Growth was monitored by chlorophyll-a content [19] that was extracted with methanol and measured by spectrophotometer at 665 nm, with the equation: C (μg ml^{-1}) = $OD_{665} \times 13.9$. Protein concentrations were measured by the Bradford assay method [5] with bovine serum albumin as the standard.

UV irradiation. A 60-W lamp (Philips, with a maximum emission at 315 nm) served as the source of UV-B (280–330 nm) [22]. Washed suspension (20 ml in a 250-ml Pyrex glass beaker) of the tested material (*Bti* spores and associated crystals or exponentially growing *Anabaena* PCC 7120 harboring pSBJ2 [32] was irradiated while agitated from a distance of 12.5 cm at the intensity of 7.5 μW cm^{-2} nm^{-1}. Aliquots were sampled at time intervals for toxicity (bioassays).

Bioassays. Eggs of *A. aegypti* were hatched in 1 L of sterile tap water supplemented with 1.5 g of Pharmamedia (Traders Protein, USA) at 30°C. Larvae of the same age and size were selected and washed for each experiment. Twenty 3rd-instar larvae, in duplicates, were incubated (28°C) in 100 ml of sterile tap water with the appropriate dilutions of cell suspensions containing the toxic proteins (irradiated or not) as necessary. Larval mortality was scored after 24 h at 28°C, and toxicity was calculated by probit analysis [12].

Viability determinations. Aliquots were appropriately diluted in sterile distilled water and evenly spread on LB (for *Bti*) or BG11 (for *Anabaena*) plates. The number of colonies was determined after 24 h or 14 days, respectively, of incubation at 30°C. No additional colonies appeared during further incubation. Each point in the survival curves is an average of duplicates in at least three different experiments.

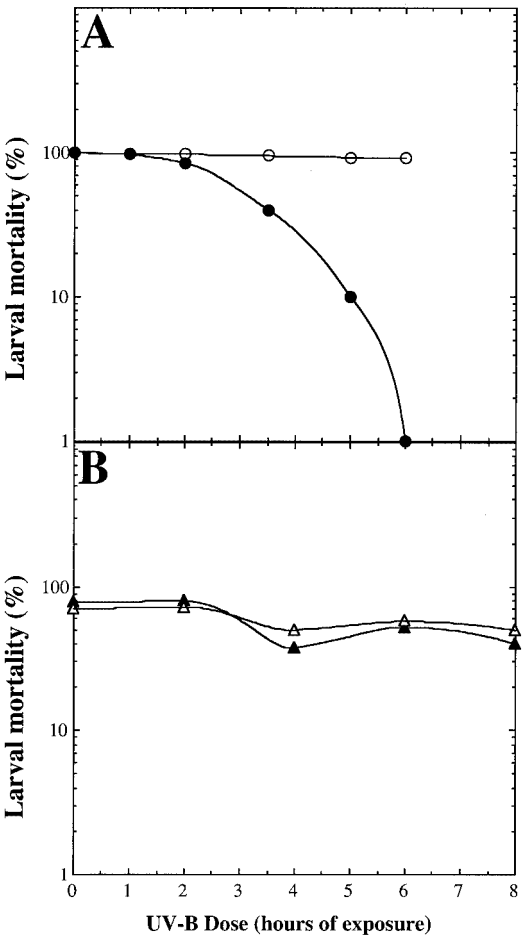

Fig. 1. Mortality of 3rd-instar *Ae. aegypti* larvae fed with: (**A**) *Bti*, UV-B-irradiated (●) or not (○); (**B**) transgenic *Anabaena* PCC 7120, UV-B-irradiated (▲) or not (△).

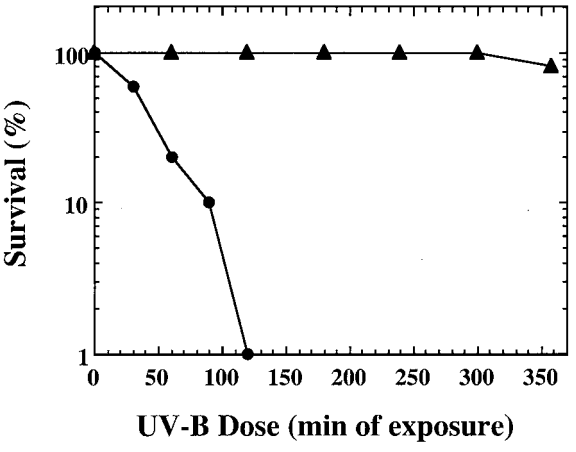

Fig. 2. Survival of UV-B-irradiated *Bti* spores (●) and recombinant *Anabaena* PCC 7120 (▲).

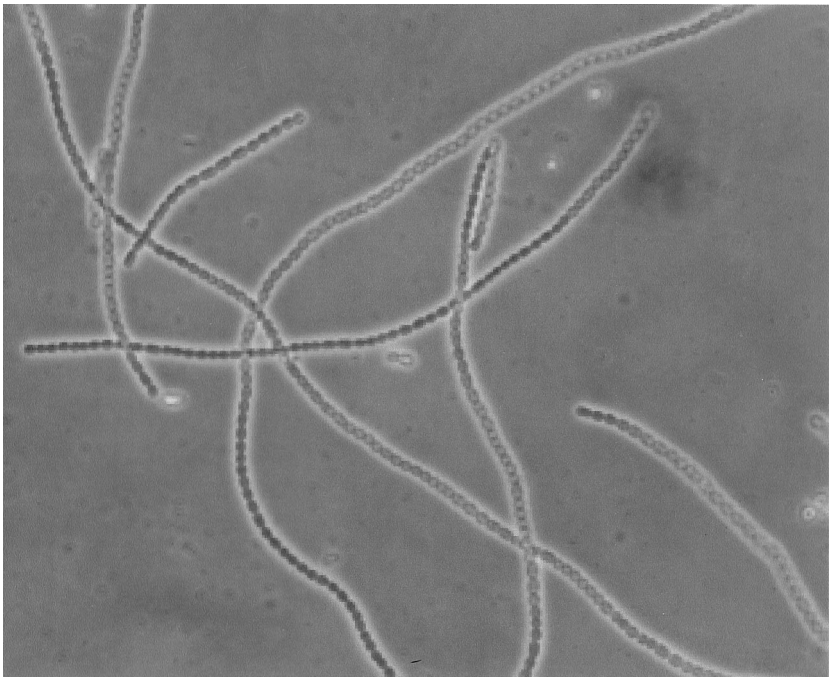

Fig. 3. Transgenic *Anabaena* PCC 7120 filamentous cells. Magnification, ×400.

Results and Discussion

Effect of UV-B irradiation on mosquito larvicidal activity was determined in suspensions of *Bti* spores and its toxic polypeptides or of transgenic *Anabaena* expressing heterologous toxin proteins.

Preliminary studies were performed to determine the concentrations of toxic (un-irradiated) cultures sufficient to kill 80–95% of larvae in an exposed population under standard conditions (LC_{80} and LC_{95}, respectively), used in further studies. Respective values were suspensions of 5×10^3 ml^{-1} for *Bti* spores and associated crystals, and 30 ng chlorophyll ml^{-1} for transgenic *Anabaena* PCC 7120.

A water suspension (20 ml in a 250-ml beaker) of exponentially growing *Anabaena* harboring pSBJ2 [32] was UV-B-irradiated while agitated. Mortality of 3-instar *A. aegypti* larvae was determined with aliquots drawn during irradiation at a cyanobacterial concentration that killed 80% of exposed population before irradiation. Similar larval mortality was obtained even after 8 h irradiation (Fig. 1B). Irradiation of water suspension of *Bti* (with spores and associated crystals), on the other hand, caused a mortality drop to 10% after 5 h (Fig. 1A). It is evident that the high toxicity of this transgenic *Anabaena* is protected from the harmful effect of UV-B and may, therefore, be protected under field conditions as well.

Viability of this *Anabaena* strain was similarly more resistant to UV-B than that of *Bti* spores (Fig. 2). This phenomenon is most likely explained by the filamentous nature of *Anabaena* PCC 7120 (Fig. 3): a single survivor among the many cells comprising a filament yields the same colony. Survival of these filaments of vegetative cells is indeed higher than that of *Bti* spores (Fig. 2), which are much more resistant to irradiation than vegetative *Bti* cells [22], being single, separate entities. Our data cannot tell which cell actually is more resistant to UV-B light since a heavily damaged chain of cyanobacteria might still count as a single colony-forming unit.

UV radiation affects many biochemical and physiological processes, including protein modification, proteolysis and enzyme inactivation. Synthesis of UV-A/B-absorbing compounds in cyanobacteria is an efficient mechanism to avoid such damage [1, 6, 10, 11, 13, 29]. For example, accumulation of phycoerythrin in the phycobilisome of *Nostoc* sp. increases the tolerance against UV-B detrimental effects owing to its protein-chromophore structure [1]. Membrane-bound pigment-protein complexes have been reported to function as UV-A/B sunscreens [6, 10]. Such elements may protect *Bti*'s δ-endotoxins expressed in the recombinant *Anabaena* PCC 7120 from damage inflicted by UV-B radiation (Fig. 1).

These results suggest an additional, potentially useful benefit of expressing *Bti* toxins in recombinant cyanobacteria. They multiply in field conditions, serve as food for mosquito larvae, and protect mosquito larvicidal toxins from UV inactivation.

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