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Comparative Sensitivity to UV-B Radiation of Two *Bacillus thuringiensis* Subspecies and Other *Bacillus* sp.

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Abstract. Susceptibility of *Bacillus thuringiensis* spores and toxins to the UV-B range (280–330 nm) of the solar spectrum reaching Earth's surface may be responsible for its inactivation and low persistence in nature. Spores of the mosquito larvicidal *B. thuringiensis* subsp. *israelensis* were significantly more resistant to UV-B than spores of the lepidopteran-active subsp. *kurstaki*. Spores of subsp. *israelensis* were as resistant to UV-B as spores of *B. subtilis* and more resistant than spores of the closely related *B. cereus* and another mosquito larvicidal species *B. sphaericus*. Sensitivity of *B. thuringiensis* subsp. *israelensis* spores to UV-B radiation depended upon their culture age; 24-h cultures, approaching maximal larvicidal activity, were still sensitive. Maximal resistance to UV-B was achieved only at 48 h.

Microbial control of agricultural pests and vectors of human diseases by *Bacillus thuringiensis* (*Bt*) is an important alternative to chemical pesticides [17, 23], but viability of spores and larval toxicity of crystals included in *Bt* preparations rapidly drop under field conditions [10, 14]. Sunlight-mediated inactivation of these preparations, which affects their efficacy and commercial value [4, 5, 11], is believed to be caused by UV damage to the spores and their δ-endotoxins [20]. Reduced exposure of *Bt* formulations to direct sunlight prolongs the efficacy of this biological control agent [19].

Insecticidal *Bt* is considered to be highly sensitive to UV radiation [6, 9, 20]. The future of *Bt* bioinsecticides depends on success to increase the resistance of spores and toxins to environmental stresses including sunlight. Most studies on molecular photobiology of spores have been carried out on the well-known genus *B. subtilis* [18, 21, 22, 24]. The effect of irradiation on viability of *Bt* spores was usually studied with the subspecies *kurstaki* (*Btk*), *galleria*, and *thuringiensis* [3–5, 11, 13], and with far-UV-light (UV-C, 254 nm), which is absorbed by the ozone layer (hence irrelevant to field conditions). Spores and vegetative cells of wild-type *Btk* were reported to be more sensitive to UV-C than those of plasmid-cured strains and of the closely related species *B. cereus* [3, 9].

Here, we compare spore sensitivity to UV-B of two important entomopathogenic bacteria, *B. thuringiensis* subsp. *israelensis* (*Bti*) and *Btk*, with those of *B. cereus* as a closely related species, *B. subtilis* as a standard, and *B. sphaericus* as another mosquito larvicidal species.

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Materials and Methods

Bacterial strains. *Bti* was isolated from a primary powder (Bactimos 1990, fun 89CO6D, Duphar B. V., Weesp, Holland). *Btk* (strain HD-1) and *B. cereus* (strain T) were kindly supplied by D. R. Zeigler (Bacillus Genetic Stock Center, Columbus, Ohio). *B. sphaericus* strains (number 2396 and 2697) were obtained from E. W. Davidson (Dept. of Zoology, Arizona State Univ., Tempe, Arizona, 85287). *B. subtilis* OI1 (*ilvCl, leu-1*) is from our collection.

Media. Luria-Bertani (LB) (0.5% yeast extract, 1% tryptone, and 1% NaCl) and modified sporulating medium (NYSM) (1% tryptone, 0.8% nutrient broth, 0.5% NaCl, 0.14 mM $CaCl_2$, 0.2 mM $MgCl_2$, 0.01 mM $MnCl_2$) [15] were used in this study.

Growth. A single colony was inoculated into a tube containing 5 ml LB and was incubated overnight at 32°C on a rotary shaker (200 rpm). 0.1 ml of the culture ($3–4\times10^8$ cells ml^{-1}) was transferred to 100 ml LB or NYSM, as indicated. Cells and spores were harvested by centrifugation at the indicated time from cultures grown under the above conditions and washed twice with sterile distilled water. Spores of both *B. sphaericus* strains used in this investigation were always produced in NYSM medium because they do not sporulate in LB medium as the other Bacillus species do. The sporulation process of *Bti*, judged microscopically, reached about 100% at 24 h in both media. Sporangia could still be detected in 48-h cultures, while 72-h cultures contained free spores only. Level of spore germination before irradiation, determined microscopically on solid LB slides, was close to 100%. No heat shock was necessary to induce germination.

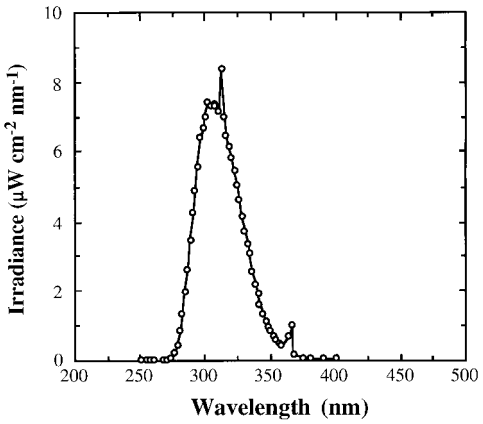

Fig. 1. Spectrum emitted by UV-B lamp (280–330 nm). The energy was measured by UV-Optronics 742 under the standard irradiation conditions (12.5 cm distance) applied in all our experiments (Materials and Methods).

UV irradiation. Spores were washed and resuspended in sterile distilled water (10^6 ml^{-1}). The transparent suspension was irradiated without agitation from a distance of 12.5 cm at the intensity of 7.5 μW cm^{-2} nm^{-1} (4.5 ml in a 40-ml beaker, 5-mm depth). The source for UV-B (280–330 nm) was a 60W Philips lamp with a maximum at 315 nm (Fig. 1). Survivors were determined by colony-forming ability at various irradiation times of up to 90 min.

Viable count. Aliquots were appropriately diluted in sterile distilled water and evenly spread on LB plates. The number of colonies was determined after 24 h of incubation at 32°C. No additional colonies appear during further incubation. Each point in the survival curves is an average of duplicates in at least three different experiments.

Results and Discussion

Photosensitivity of *B. thuringiensis* subsp. *israelensis* spores. *Bti* displayed maximum larvicidal activity after 24–27 h growth in LB broth at 32°C [2], while spores were still very sensitive to UV-B (Fig. 2). Maximal resistance was acquired at 48 h, when mature spores were observed by phase-contrast microscopy. This may be attributed to the physiological state of the spores: at 24 h, they are still detected in sporangia, contain water, and are not always in complete maturation. At 48 h, spores are found in the dormant state and no longer contain water. It is known that the water is the most important factor in determining heat resistance of spores [22]. All the following experiments were, therefore, conducted with 48-h cultures. Photosensitivity of freshly prepared spores seems not to be affected by the composition of the medium (LB or NYSM), but spores of the commercial powder were more sensitive (data not shown).

Comparative photosensitivity of various *Bacillus* ssp. spores. Spores of various *Bt* subspecies producing Cry insecticidal protoxins are considered to be more sensitive to UV-C radiation (254 nm) than acrystalliferous mutants and other bacillus species [3, 5, 7, 9]. Tested with UV-B (reaching Earth's surface), spores of *Bti* were as resistant as spores of *B. subtilis* and more resistant than those of *B. cereus*, *B. sphaericus*, and *Btk* (Fig. 3). The reason for this difference is not obvious. Both *Bti* and *Btk* produce, during sporulation, Cry protoxins with similar

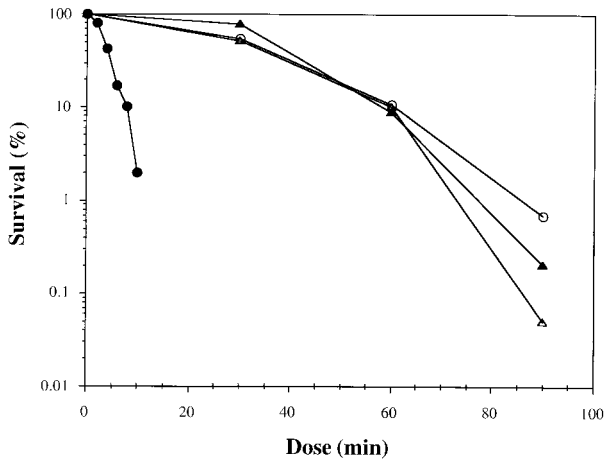

Fig. 2. Dependence of UV-B sensitivity of *B. thuringiensis* subsp. *israelensis* spores on age of spore culture. Washed cells and spores of *Bti*, grown under the conditions described in Materials and Methods in LB medium for 24 h (●), 48 h (▲), 72 h (△) and 96 h (○), were UV-B irradiated for various periods of time. Survivors were scored by viable counting.

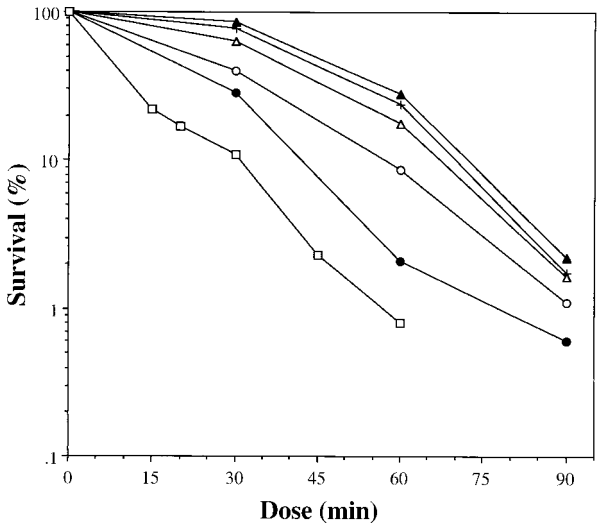

Fig. 3. UV-B sensitivity of spores of *B. thuringiensis* subsp. *israelensis* (+), *B. thuringiensis* subsp. *kurstaki* (□), *B. cereus* (●), *B. subtilis* (▲) and *B. sphaericus* 2697 (△) and 2396 (○). Freshly prepared spores were grown for 48 h at 32°C in LB, except *B. sphaericus* strains in NYSM. Irradiation conditions and scores for survivors were as described in Materials and Methods.

molecular weights that are crystallized into parasporal inclusions [12] and contain a similar number of plasmids (11 in *Btk*, 9 in *Bti*) [3, 17]. The rates of target killing, on the other hand, differ significantly: *Bti*'s Cry toxins kill mosquito larvae within hours [17], whereas *Btk*'s anti-lepidopteran toxins act much more slowly and with synergistic contribution of their spores [9, 16]. The synergistic effect of *Btk* spores depends on the existence of Cry1A protoxins in the spore coat, while the spore surface of *Bti* contains very little, if any, of the protoxins, [1, 9]. The excess of Cry1A in *Btk*'s spore coat is compensated by a deficiency of low-molecular-weight *B. cereus*-like spore coat polypeptides, a deficiency not found in *Bti* [1]. Replacement of low-molecular-weight proteins by Cry protoxins in the spore coat may influence its structure because the association of protoxin is loose, altering the physiology and resistance properties of the spore in many ways [1, 9]. This may be the reason that *Btk* spores are more sensitive to UV-B than are *Bti* spores (Fig. 3).

Plasmid content also affects UV sensitivity of *Bt* spores [3, 7], but sensitivities of strains HD-1 and HD-73 of *Btk* are similar despite the large difference in the number of their plasmids (11 and 5 respectively) [3]. The two strains, however, similarly deposit Cry1A protoxins in their spore coat [1, 9].

Another factor responsible for UV resistance is the high concentrations of small acid-soluble proteins (SASP) [18, 22]. Spores of different *Btk* strains and of *B. cereus* contained SASP similar to those of *B. subtilis* [3]. SASP of *Bti* and *B. sphaericus* spores are less abundant and run differently on SDS-PAGE than *B. subtilis* spores [8], observations that might explain at least partially the higher sensitivity of their spores to osmotic pressure and UV light [6, 7]. Nevertheless, in our experiments, spores of *Bti* and *B. subtilis* were similarly resistant to UV-B (Fig. 3). SASP contribution to UV-resistance thus seems not to be able to explain the observed difference in UV-B sensitivity.

Susceptibility of *B. subtilis* spores to solar radiation can be attributed to the fact that sunlight is composed of radiation in the whole range between 290 nm to above 780 nm rather than UV-C (254 nm) [24]. It has recently been demonstrated that spores of *B. subtilis* mutants defective in spore coat layers were more resistant to UV-C, but significantly more sensitive to sunlight, to UV-B (290-320 nm), and to UV-A (320–390) than their wild-type parental strain [21]. These data indicate that the spore coat, particularly its inner layer, plays a role in spore resistance to environmentally relevant wave length [21]. Differences in sensitivity to UV-B between *Btk* and *Bti* spores may thus be coupled to a difference in the structure of the spore coat layers between these microorganisms [1].

Spore resistance to organic solvents, heat, enzymes (lysozyme), desiccation, water, and some other treatments is also a function of spore coat [22]. For example, sensitivities to hyperosmotic stress of *Btk*, *Bti*, and *B. cereus* spores to 1 M NaCl on LB plates were compared by counts of colony forming units; *Bti* spore survival was 70–75%, *B. cereus*'s was between 6 and 8%, and *Btk*'s dropped to below 0.1% (unpublished observations). Low osmotolerance of *Btk* spores might also be explained by structural and physiological variation in spore coat layers.

Levels of resistance to UV-B of spores from different Bacillus species appear to be related to the quantity and quality of SASP and to activities of DNA repair systems [7, 8, 18, 21, 22, 24]. Imperfection of spore coat may influence the photochemistry through conformation of DNA and activity of repair pathways, which may consequently lead to high UV-B sensitivity of *Btk* spores. However, too little is known about the effects of UV-B and UV-A on spore coat components and about their targets.

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