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Plasmid-Associated Sensitivity of *Bacillus thuringiensis* to UV Light

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Spores and vegetative cells of *Bacillus thuringiensis* were more sensitive to UV light than were spores or cells of plasmid-cured *B. thuringiensis* strains or of the closely related *Bacillus cereus*. Introduction of *B. thuringiensis* plasmids into *B. cereus* by cell mating increased the UV sensitivity of the cells and spores. Protoxins encoded by one or more *B. thuringiensis* plasmids were not involved in spore sensitivity, since a *B. thuringiensis* strain conditional for protoxin accumulation was equally sensitive at the permissive and nonpermissive temperatures. In addition, introduction of either a cloned protoxin gene, the cloning vector, or another plasmid not containing a protoxin gene into a plasmid-cured strain of *B. thuringiensis* all increased the UV sensitivity of the spores. Although the variety of small, acid-soluble proteins was the same in the spores of all strains examined, the quantity of dipicolinic acid was about twice as high in the plasmid-containing strains, and this may account for the differences in UV sensitivity of the spores. The cells of some strains harboring only *B. thuringiensis* plasmids were much more sensitive than cells of any of the other strains, and the differences were much greater than observed with spores.

In general, *Bacillus* spores are considerably more resistant to UV light than are the vegetative cells (8). This resistance is due primarily to an altered conformation of the DNA and hence the types of photoproducts produced (8). These unique photoproducts apparently are not as damaging as those produced in vegetative cells and perhaps are more readily repaired (11). The conformation of DNA in spores is partly dependent upon the presence of several small, acid-soluble proteins (SASP) (18, 23, 26). Spores produced by *Bacillus subtilis* mutants unable to synthesize certain SASP are very sensitive to UV light (18). Another factor which may contribute to spore UV sensitivity is the quantity of dipicolinic acid (DPA) deposited in the spore (10).

It has been noted that following the application of *Bacillus thuringiensis* preparations containing spores and inclusions to control insects, there is a fairly rapid disappearance of *B. thuringiensis* (9). This lack of persistence has been attributed in part to light sensitivity (including UV) of *B. thuringiensis* spores (12). Indirect comparisons indicate that *B. thuringiensis* spores are more sensitive than those produced by the very closely related species *Bacillus cereus* (5, 28).

Although the factors mentioned above could contribute to the apparent UV sensitivity of *B. thuringiensis* spores, *B. thuringiensis* has other properties which may play a role. These include the relative abundance of plasmids in *B. thuringiensis* isolates and the possibility that some factors encoded by these plasmids, such as protoxins (7, 25), could influence UV sensitivity. Some protoxin remains bound to the spore surface (2, 3) and might alter the light sensitivity. Alternatively, the presence of plasmids may affect spore resistance to UV light, possibly by altering the variety of SASP (23) or the amount of DPA (10) in the spore.

The existence of *B. thuringiensis* strains with various numbers of plasmids (Cry^+ or Cry^-), as well as the ability to transfer *B. thuringiensis* plasmids to *B. cereus* (1, 7) or to introduce plasmids directly into *B. thuringiensis* (21), prompted a reinvestigation of *B. thuringiensis* spore and cell properties which may contribute to UV sensitivity. It appears that plasmids increase the UV sensitivity of spores, perhaps by increasing the quantity of DPA. The presence of *B. thuringiensis* plasmids increases the sensitivity of vegetative cells even more dramatically than that of spores.

MATERIALS AND METHODS

Bacterial strains, spore production, and spore purification. The properties of the bacterial strains used are summarized in Table 1. Spores were produced by plating late-log-phase nutrient broth cultures grown at 32°C on nutrient agar and incubating them at 32°C for 4 to 5 days. Some spores of strain HD-1-9 were produced at 25°C. Cells of strain CryB (pXI93) and CryB(pBC16) were grown in nutrient broth containing 40 μg of tetracycline per ml, and those of strain CryB(pHP13) were grown in nutrient broth containing 5 μg of chloramphenicol per ml. Spores and inclusions harvested from plates were washed once with distilled water and then dispersed in 0.1% (vol/vol) Triton X-100 by gentle agitation for 15 to 18 h. Dispersed spores were purified by centrifugation through 67% (vol/vol) Renografin (E. R. Squibb & Sons) (24) at 35,000 × *g* for 35 min at 25°C. Purified spores were washed twice with distilled water, checked microscopically for phase brightness, and used immediately.

UV irradiation. Purified spores were diluted and irradiated as 1-ml suspensions in open plastic petri dishes resting below a Sylvania model G 30T8 30-W germicidal lamp with a constant output of 8 J/m^2 per s at spore level. More than 90% of the energy was emitted at 253.7 nm. Spores were irradiated for times up to 120 s, suspended in situ in molten (45°C) nutrient agar, and incubated for 24 h at 37°C for measurement of viability. To measure photoreactivation, some irradiated spores were incubated under fluorescent lights while others were incubated in the dark. At least seven individual spore preparations of each strain were tested.

Cells of the strains listed in Table 1 were grown in 3 ml of

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TABLE 1. Bacterial strains and plasmids

Bacterial strain and plasmid	Description[a]	Source or reference
B. cereus		
569	Str^r	19
C_{1-4}	Mating of *B. thuringiensis* subsp. *kurstaki* HD-73 and *B. cereus* 569 Str^r. Contains plasmids of 50 and 4 to 5 MDa from *B. thuringiensis* subsp. *kurstaki* HD-73 and produces CryIA(c) protoxin.	19
B. thuringiensis		
subsp. *kurstaki* HD-1	Contains 11 plasmids including those encoding three CryI and two CryII protoxins	Isolated from Dipel (Abbott Laboratories)
subsp. *kurstaki* HD-73	Contains five plasmids and produces the CryIA(c) protoxin	BGSC[b]
subsp. *kurstaki* HD-1-9	Plasmid-cured derivative (loss of six) of strain HD-1. Forms CryIA(b) protoxin, which is stable at 25°C but not 30°C.	19
CryB	Acrystalliferous derivative of strain HD-1 derived by heating spores. Contains only one plasmid of >130 MDa.	25
Mit6	Acrystalliferous derivative of strain HD-1 derived by treatment with mitomycin C. Contains only plasmids of 4.5 and 5 MDa.	1
B. thuringiensis transcipients		
CryB(pHP13)	Cm^r Em^r. Contains a 4.9-kb plasmid.	13
CryB(pBC16)	Tc^r. Contains a 4.6-kb plasmid.	6
CryB(pXI93)	Clone of *cryIA(b)* gene (*Bam*HI-*Pst*I fragment) in pUC8-pBC16 shuttle vector). Contains a 10-kb plasmid.	21

[a] Just before use, each of the strains listed was verified for plasmid composition consistent with the description by the methods previously described (19).
[b] BGSC, Bacillus Genetic Stock Center, The Ohio State University, Columbus.

LB broth (plus 40 μg of tetracycline per ml or 5 μg of chloramphenicol per ml when appropriate) at 30°C for 4 h (mid-log phase). Cells were collected by centrifugation at 4,000 rpm for 10 min in a Sorvall SS-1 rotor and suspended in 7 ml of 0.01 M $MgSO_4$ (4, 22) to about 1×10^7 to 2×10^7 per ml in sterile glass petri dishes. The cells were exposed to a 254-nm lamp with or without a reduced aperture (output of 3.4 or 0.6 J/m^2 per s) for various times and sampled into dilution fluid (0.1% tryptone plus 0.5% NaCl). All dilutions were plated on G-Tris agar (with or without antibiotics) (1) and incubated at 30°C in the dark. Duplicate sets of some dilutions were incubated at 27°C under fluorescent lights. Each experiment was performed three times, and the averages were plotted.

Spore coat removal. Spore coats were removed to assess the role of crystal protein in spore inactivation. Coats were extracted from spores with a solution containing 0.5% sodium dodecyl sulfate, 0.1 M dithiothreitol (pH 10.0), and 1.0 M NaCl during a 2-h incubation at 37°C. Extracted spores were washed twice with distilled water, and their viability was measured by plate count. Removal of spore coats was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracts (3, 27) and by testing for the transition of spores from phase bright to dark after treatment with 1 mg of lysozyme per ml.

Assay of DPA. Washed, purified spores were dried under vacuum and weighed to produce a measurement of the number of milligrams of spores per milliliter of suspension. DPA was assayed by the method of Janssen et al. (15) as modified by Rotman and Fields (20), and the number of micrograms of DPA per milligram of spores was calculated.

Analysis of SASP. The SASP were extracted from spores by the method of Tipper et al. (26). Spores were incubated in 2 N HCl for 30 min at 25°C to bring about "acid popping" (A. D. Warth, Spore Newsl. 6:4–6, 1979) and release of the SASP. Debris were removed from the lysate by centrifugation at 16,000 × *g* for 10 min. The extract was electrophoresed in 10 to 20% linear gradient sodium dodecyl sulfate-polyacrylamide gels, and proteins were stained with silver (29). Acid-soluble extracts of *B. subtilis* 168 spores were electrophoresed for confirmation of the α,β size class of SASP (26) in the *B. cereus* and *B. thuringiensis* samples.

RESULTS AND DISCUSSION

This study began with the observation that *B. thuringiensis* subsp. *kurstaki* HD-1 spores were more sensitive to UV irradiation than were *B. cereus* 569 spores, with a typical 1.7- to 2-fold difference in the slopes of survival curves (Fig. 1) and a 5- to 6-fold difference at the highest dose (Table 2). An even greater difference was found for vegetative cells of these two species with much lower UV doses (Fig. 2). Two possibilities were initially tested, i.e., that the differences were due to (i) the presence of plasmids in *B. thuringiensis* or (ii) differences in the capacity to photoreactivate.

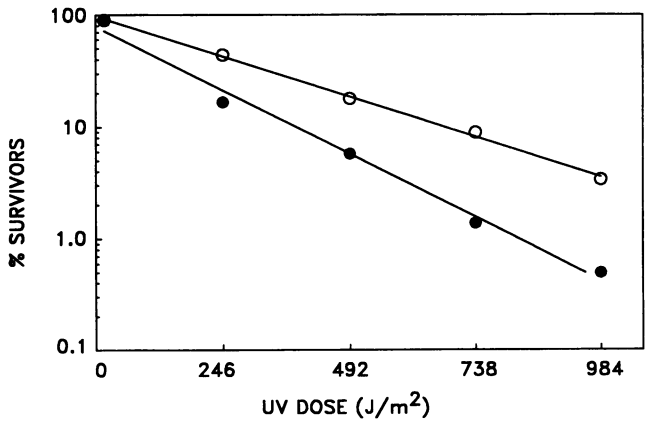

FIG. 1. UV inactivation of *B. cereus* (○) and *B. thuringiensis* subsp. *kurstaki* HD-1 (●) spores. Spores were irradiated, and their viabilities were measured as described in Materials and Methods. The results show the typical 1.7- to 2-fold difference in slopes observed in all trials.

Spores from two plasmid-cured, acrystalliferous derivatives of *B. thuringiensis* subsp. *kurstaki* HD-1 (Mit6 and CryB) displayed decreased sensitivity to UV inactivation compared with spores of the parental strain. The results with the plasmid-cured *B. thuringiensis* strains were similar to those obtained with *B. cereus* 569 (Table 2). On the other hand, spores of the crystal-forming *B. cereus* transcipient, C_{1-4}, were more sensitive to UV inactivation than were spores of the parental strain. In this case, the results were similar to those obtained with *B. thuringiensis* subsp. *kurstaki* HD-73 (Table 2), the plasmid donor for strain C_{1-4} (Table 1). No appreciable photoreactivation of any of the spores was observed. The results were consistent with the conclusion that the presence of a protoxin-encoding plasmid from *B. thuringiensis* subsp. *kurstaki* HD-73 and perhaps *B. thuringiensis* subsp. *kurstaki* HD-1 is responsible for the differences in sensitivity. In fact, introduction of a cloned protoxin gene [*cryIA(b)*] into strain CryB on a high-copy-number shuttle vector [strain CryB(pXI93)] increased the UV sensitivity of the spores (Table 2).

The spores of crystal-forming and non-crystal-forming strains differ in the nature of the proteins present in the spore coat, with the former having comparatively thin coats plus a deposit of protoxin protein and the latter having thicker spore coats (comparable to those of *B. cereus*) that lack protoxin (3, 28). The differences reported in Table 2 may therefore have been due to the presence of protoxin on the coats of the more UV-sensitive spores. It was conceivable that during irradiation protoxin molecules were cross-linked to other proteins in the coat and thus interfered with germination. If so, spores might be made less sensitive by removing the coats before or after irradiation. None of the spores tested, however, were rendered less sensitive to UV by removing the spore coat before irradiation, nor could they be rescued from inactivation by removing the spore coat after irradiation. In addition, spores of *B. thuringiensis* subsp. *kurstaki* HD-1-9 were equally sensitive whether produced at the permissive or nonpermissive temperature for protoxin accumulation (Table 2).

The results of these experiments suggested that the UV sensitivity of *B. thuringiensis* spores was not due to the protoxin on spore coats, even though strain CryB(pXI93), which contained a cloned protoxin gene, was very sensitive to UV light (Table 2). Since only a 4.4-kilobase (kb) fragment containing the *cryIA(b)* gene was inserted into the cloning vector to construct pXI93, it is unlikely that any other *B. thuringiensis* plasmid-encoded product was involved. Two additional CryB transformants containing the low-copy-number plasmid pHP13 (13) or the high-copy-number pBC16 (6) were more sensitive to UV inactivation than strain CryB was (Table 2). Not surprisingly, strain CryB(pBC16) was about as sensitive as strain CryB(pXI93), since the cloning vector used for pXI93 is a chimera of pBC16 and pUC8 (21). These results further confirm that UV sensitivity of *B. thuringiensis* spores is not due to a specific *B. thuringiensis* plasmid gene or gene product.

TABLE 2. UV inactivation of spores from strains of *B. cereus* and *B. thuringiensis*

Organism[a]	% Survival[b]
Spores with low UV sensitivity	
B. thuringiensis CryB	4.3 ± 2.2
B. thuringiensis Mit6	3.9 ± 0.8
B. cereus 569	3.7 ± 1.6
Spores with high UV sensitivity	
B. thuringiensis subsp. *kurstaki* HD-1	0.7 ± 0.4
B. thuringiensis subsp. *kurstaki* HD-73	0.5 ± 0.3
B. thuringiensis subsp. *kurstaki* HD-1-9 (25°C)	0.9 ± 0.4
B. thuringiensis subsp. *kurstaki* HD-1-9 (32°C)	0.9 ± 0.4
B. cereus C_{1-4}	0.8 ± 0.1
B. thuringiensis CryB(pHP13)	0.5 ± 0.03
B. thuringiensis CryB(pBC16)	0.2 ± 0.07
B. thuringiensis CryB(pXI93)	0.1 ± 0.03

[a] The placement of each organism in one or the other group was established as statistically correct by using Tukey's studentized range test.
[b] After irradiation with 984 J/m^2. See Fig. 1 and Materials and Methods.

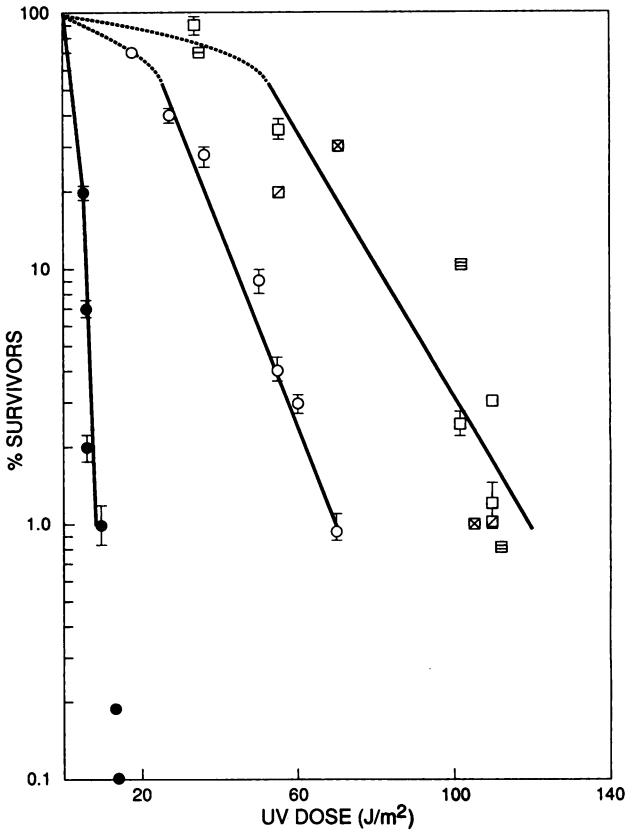

FIG. 2. UV inactivation of vegetative cells of *B. thuringiensis* CryB (□), *B. thuringiensis* CryB(pHP13) (▨), *B. cereus* 569 (⊠), *B. thuringiensis* Mit6 (▤), *B. thuringiensis* CryB(pBC16) (○), and *B. thuringiensis* subsp. *kurstaki* HD-73 (●). Virtually identical results were obtained with *B. thuringiensis* subsp. *kurstaki* HD-1, HD-1-9, and the transcipient *B. cereus* C_{1-4} as were found with *B. thuringiensis* subsp. *kurstaki* HD-73.

At least two constituents of spores are known to be involved in sensitivity to UV light: SASP and DPA. The spores of *B. subtilis* strains unable to synthesize certain SASP are extremely sensitive to UV light (18, 26), whereas *B. cereus* spores exhibit increased sensitivity with increased quantities of DPA (10). The spores of the strains listed in Table 1 contained SASP bands which electrophoresed at the same positions as the SASP of *B. subtilis* 168. Spores of plasmid-cured strains, however, contained about half as much DPA as did the corresponding plasmid-containing strains (Table 3), so within each species there was a correlation between UV sensitivity and DPA content. This correlation is consistent with previous observations (10), but the reason why plasmid-containing strains should have more DPA per spore is not known.

TABLE 3. DPA content of spores from strains of *B. cereus* and *B. thuringiensis*

Organism	DPA content (% [dry wt] of spores)
B. thuringiensis strains and derivatives	
subsp. *kurstaki* HD-73	19.1 ± 1.4
subsp. *kurstaki* HD1-9	13.3 ± 1.4
CryB	6.9 ± 3.2
CryB(pHP13)	15.3 ± 2.5
CryB(pBC16)	15.8 ± 2.2
B. cereus and a transcipient	
569	2.8 ± 0.6
C_{1-4}	4.9 ± 0.5

In contrast to spores, cells with no (*B. cereus*) or few (*B. thuringiensis* Mit6 and CryB) plasmids or with the low-copy-number plasmid pHP13 were fairly resistant to UV light, but were still about 10-fold more sensitive than spores (Fig. 2). Cells of CryB (pBC16) had an intermediate resistance, whereas *B. thuringiensis* cells with several indigenous plasmids or a *B. cereus* transcipient containing plasmids from *B. thuringiensis* subsp. *kurstaki* HD-73 (i.e., strain C_{1-4}) were very sensitive. The cells containing indigenous *B. thuringiensis* plasmids were almost as sensitive as mutagenic repair-deficient *Escherichia coli* cells (22), even though some photoreactivation was found for these *B. thuringiensis* cells which had been exposed to a UV dose of 8 to 15 J/m^2. These *B. thuringiensis* strains were much more sensitive to UV light than were cells from a *B. thuringiensis* subsp. *thuringiensis* strain of unknown plasmid composition (4) but were only slightly more sensitive than cells of *Bacillus anthracis* which contain plasmids (17). Interestingly, *B. subtilis* 168 without plasmid pBC16 was slightly more sensitive to UV light than was *B. subtilis* 168(pBC16), but the difference, if any, was much smaller than for the comparable *B. thuringiensis* CryB strains (Fig. 2).

It is known that *B. thuringiensis* subsp. *kurstaki* HD-1 and the derivative, HD-1-9, carry at least one prophage (TP21) in the form of a 29-megadalton (MDa) extrachromosomal circulator molecule (C. Thorne, personal communication). During late-exponential growth, this prophage is converted to a transducing phage. Perhaps one or more additional prophages exist as, or in, the *B. thuringiensis* plasmids of the strains tested (similar to *B. thuringiensis* AF101 [16]), and their induction could be lethal. UV light can induce bacteriophage production in *B. thuringiensis* subsp. *aizawai* (14) and in *B. thuringiensis* subsp. *kurstaki* HD-1 as measured by lysis of *B. cereus* 569 (A. Aronson, unpublished results), so a major reason for the extreme sensitivity of these plasmid-bearing strains could be phage induction. One exception to this extreme sensitivity was *B. thuringiensis* subsp. *finitimus*, which contains only two very large plasmids and has a UV sensitivity similar to that of CryB(pBC16) in Fig. 2 (Aronson, unpublished).

Finally, it should be noted that if similar effects were observed when using other inactivating wavelengths for *B. thuringiensis* spores, notably 330 and 400 nm (12), an opportunity to improve the field survival of commercial *B. thuringiensis* strains may be possible. It is assumed that the loss of cryptic plasmids would not have a detrimental effect on the growth or survival of *B. thuringiensis* strains. If so, moving protoxin genes to the chromosome of a plasmid-cured strain might add resistance and hence survivability to the spores and cells without loss of the capacity to produce a toxic inclusion.

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