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Development of Ultraviolet Resistance in Sporulating *Bacillus cereus* T

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Ultraviolet (UV) resistance patterns of sporulating *Bacillus cereus* were determined. Six different categories of UV resistance were discernible as the organism progressed from exponential growth to the free, mature spore. Light microscope observations allowed the assignment of certain sporulation stages to specific UV resistance categories. Marked changes in UV resistance were associated with stages III, mid-IV, and post-IV of sporulation. Dipicolinic acid was shown to sensitize forespores to UV radiation. Mechanisms invoked to explain the different UV categories are discussed.

The bacterial endospore typically exhibits greater ultraviolet light (UV) resistance than does the vegetative form of the same species (11, 14). Resistance to UV inactivation appears about midway in the sequence of sporulation events (11, 15). Studies to date, however, have relied on the determination of the survivors of a single UV dose which was sufficient to inactivate vegetative forms but not spores (11, 15). Although this approach clearly gives the time of appearance and increase in the population of forms resistant to the particular UV dose used, it does not allow one to observe whether changes occur in the UV inactivation patterns of sporulating bacteria. Since sporulation involves a host of sequential morphological and biochemical changes, one might expect the UV resistance patterns to be complex and perhaps of some value in increasing our understanding of this differentiation process.

We have reported that the content of dipicolinic acid (DPA) in mature spores affected their UV survival. Surprisingly, spores lowest in DPA content were most resistant to UV (<280 nm) inactivation (5), the DPA sensitizing spore deoxyribonucleic acid (DNA) to UV. It may be deduced from Vinter's studies on sporulation (15) that the time of DPA accumulation overlaps with that for the appearance of forms resistant to a single UV dose. Thus, one might expect that during sporulation, at a time before, or early in DPA synthesis the culture would exhibit greater UV resistance than the mature spore. Our studies support such a notion and give detailed information on the development of UV resistance in sporulating cultures.

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MATERIALS AND METHODS

Bacteria. Two strains of *Bacillus cereus* T were used: a wild-type laboratory strain (obtained from Harlyn Halvorson) and HW-1, a DPA$^-$ mutant (5, 7, 9, 17, 18) obtained from H. Orin Halvorson. Spores of the mutant strain contained less than 1% wild-type DPA level (5). Mutant spores containing up to 4% DPA (dry weight) could be produced by the addition of appropriate amounts of DPA (100 μg/ml) to the culture at stage II of sporulation.

Media. Bacteria were grown and sporulated in modified G medium (13) and enumerated by the pour plate method in a nutrient agar (beef extract, 3 g; peptone [Oxoid], 5 g; agar [Difco], 15 g; per liter of water). All dilutions and washes used 0.05 M NaK phosphate buffer, pH 7.

Light microscopy. Culture samples for determination of the stages of forespore development were chilled in ice and examined immediately by the method of Gordon and Murrell (6). Suitable groups of chains (see Fig. 1b of reference 6) were examined for (i) the number of cells in each chain, (ii) the number of forespores in each chain, and (iii) the stage(s) of development of each forespore in each cell of the chain. Fields from different parts of the slide were examined until a total of 80 to 100 chains (>300 cells) were classified. With experience, the following stages of forespore development could be readily identified and quantitated—stage II, straight-to-curved septa; stage III, forespore incompletely engulfed and seen as a small, oval, stained area at the extreme tip of the mother cell; early stage IV, forespore completely engulfed, non-refractile, and seen as a much larger stained, oval area extending into the middle of the mother cell. The next stage, mid-stage IV, was

observed with Zeiss phase-contrast optics. Two morphological classes were distinguished in this stage, (i) phase-brown forespore and (ii) phase-white forespore (refracted light not greater than slide background). Finally, refractile forespores (RFS; refracted light greater than slide background) were also enumerated. A constant light setting was used for phase-contrast observations. Typical forespore stages discussed above are illustrated in Fig. 1.

UV irradiation. Culture samples were diluted at least 1/500 into 15 ml of diluent and irradiated in petri dishes (300 ergs per mm^2 per min) with vigorous stirring. An 8-W germicidal lamp (principal output at 254 nm) was used. The 254 nm absorption of the irradiated suspensions was always less than 5%/cm. Plated irradiated samples were enumerated after 24 h of incubation in the dark at 30 C. All irradiations and plating were done in dim light (no fluorescent lights).

DPA analysis. Culture samples were centrifuged, the pellet washed once with diluent, and the DPA in the washed pellet was extracted and estimated by the method of Lewis (8).

UV parameters. UV inactivation curves are described in terms of shoulder, D_{10} (exp) and extrapolation number, n. D_{10} (exp) is used specifically to describe the dose which will reduce the survivors by 90% over the exponential part of the curve. n is the value obtained by extrapolating this part of the curve to the ordinate and correcting for the proportion of the population (indicated by the plateau level, see below) contributing to the exponential decline. For monophasic inactivation curves, the shoulder is obtained by extrapolating the exponential part of the curve to the 100% survivor level. For biphasic curves, the shoulder, henceforth called the plateau, is measured by extrapolating the exponential phase to the plateau level below the 100% survivor level.

Statistical analysis. A regression of the log of percent survivors (v) on dose (x) was calculated for each set of data using the equation:

$$Y = a + \frac{b}{2}(x - s) + \frac{b}{2}\sqrt{(x - s)^2 + c}$$

This represents two straight lines (the first horizontal at $y = a$, and the second with slope b with a smooth transition between them (curvature parameter c) at the shoulder were $x = s$. The value of a was assumed to be log 100 for monophasic curves and was estimated for biphasic curves by excluding data points on the first phase. The equation is adapted from one suggested by Griffith and Miller, (accepted for *Contributions in Statistics*, 1973) and was fitted by least-squares using the procedure described by Nelder and Mead (10).

RESULTS

Characteristics of the system. *B. cereus* grows and sporulates in chains. Thus, any changes observed in the UV survival curves of sporulating cultures could be due to (i) changes in the average chain length, (ii) changes in the proportions of forespores within the chains, in addition to (iii) fundamental changes in the UV sensitivity of forespores during development into mature, free spores. In the studies reported here, we have monitored the morphological development of sporulating cultures at each UV sampling. Since sporulation is not a highly synchronous process, most of the UV-irradiated populations were morphologically heterogeneous. Thus, in any particular sample, some chains were more advanced in sporulation than others. Therefore, if differences in UV resistance exist among the different stages of sporulation, such populations are not expected to yield

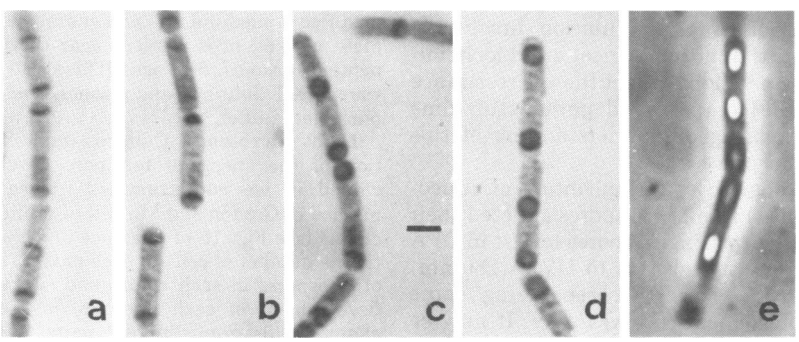

FIG. 1. *Light micrographs of sporulation stages of B. cereus T. (a–d) Crystal violet stain; (e) phase contrast. (a) Stage II: septa (darkly stained) are straight, or slightly curved. (b) Stage II, more advanced than (a). Septa are definitely rounded, and volume of cytoplasm in septated segment is increasing. (c) Stage III: engulfment is incomplete, forespore (darkly stained) is increasing in size. (d) Early stage IV: forespore appears as an oval body which has moved away from the end of the cell. Photograph shows three early stage IV and two stage III forespores (second and third from bottom). (e) Late stage IV and refractile forespores (RFS). Under phase-contrast microscopy, the dark bodies (early stage IV) gradually lighten to brown, then white, and then become refractile. Three refractile and two phase white forespores are shown. For details of photographic methods see reference 6.*

simple monophasic UV inactivation curves (12). The observations reported here rely on the recognition of such biphasic UV inactivation patterns and their relationship to specific stages of spore morphogenesis. As will be seen later, chain length did not appear to affect the UV survival of sporulating cultures (Tables 1 and 2, Fig. 2 and 3).

Log-phase cells to stage II. UV survival curves, population morphology, and UV resistance parameters for an exponentially growing culture and a culture in various early stages of sporulation are given in Fig. 2 and Table 1. The survival curve of the log-phase culture (Fig. 2a, curve 1) had a shoulder which extended to 85 ergs/mm^2 followed by an exponential decline in colony-forming units at a rate of one order of magnitude for every 51 ergs/mm^2, i.e., D_{10} (exp) = 51 ergs/mm^2 (Table 1). Other experiments (not shown) indicated that, as cultures left the exponential phase and entered stationary phase, the shoulder increased to about 120 ergs/mm^2, whereas the D_{10} (exp) remained unchanged. This increase in shoulder was not accompanied by an increase in average chain length. It has been suggested (12) that cultures in conditions of unbalanced growth exhibit longer shoulders than in balanced (exponential) growth.

No further change in UV resistance was evident between late stationary-phase cells and stage II of spore formation. The extrapolation number, n, was approximately 800.

Stage III and stage IV. The survivor curve

TABLE 1. *Morphology and UV resistance of exponential-phase and early sporulating B. cereus T*

Time[a] (t h)	Curve[b]	Percentage of chains with at least one of the forespore forms below				No. of cells per chain	UV parameter		
		II	III	IV			Shoulder (ergs/mm^2)	Slope[c]	D_{10} (exp) (ergs/mm^2)
				Early	Mid				
−0.6	1	—[d]	—	—	—	4.3	85	−0.019	51
4.5	2	89	0	0	0	4.1	120	−0.016	64
7.75	3	—	86	43	0	3.5	50	−0.014	73
9.1	4	—	—	$\leqslant$55	34	3.4		−0.005	212[e]
8.2	5[f]	—	—	—	16	4.2		−0.008	132[e]

[a] t_0 = end of exponential-phase of growth and beginning of sporulation time scale.
[b] Refer to Fig. 2 for inactivation curves.
[c] The pooled standard error was less than ±0.003 in all cases.
[d] Not determined.
[e] Represents only most resistant fraction.
[f] Zero-hour sample of Fig. 3a and Table 2.

TABLE 2. *Morphology and UV resistance of B. cereus T during late stages of sporulation*

Hours	No. of cells per chain	Percentage of chains with a refractile forespore	Percentage of high-resistance class	UV parameter of high-resistance class[a]		
				Plateau (ergs/mm^2)	Slope[b]	D_{10} (exp) (ergs/mm^2)
0[c,d]	4.2	About 1	0	—[e]	—	—
1[c]	3.0	18	16	300	—	—
1.5	3.8	45	35	300	−0.00097	362
2[c]	3.2	51	41	290	−0.0010	371
2.5	4.0	71	49	360	−0.0010	369
3[c]	4.6	74	66	300	−0.0009	495
4	3.7	88	90	280	−0.0013	398
6	4.1	100	100	360	−0.0016	321
10[c]	3.4	100	100	340	−0.0019	264
26[c]	1.0	Free spores	100	250	−0.0026	190

[a] See text for description of this class.
[b] The pooled standard error was less than ±0.00056 in all cases.
[c] Refer to Fig. 3 for inactivation curve.
[d] 0 h = $t_{8.2}$.
[e] Not determined.

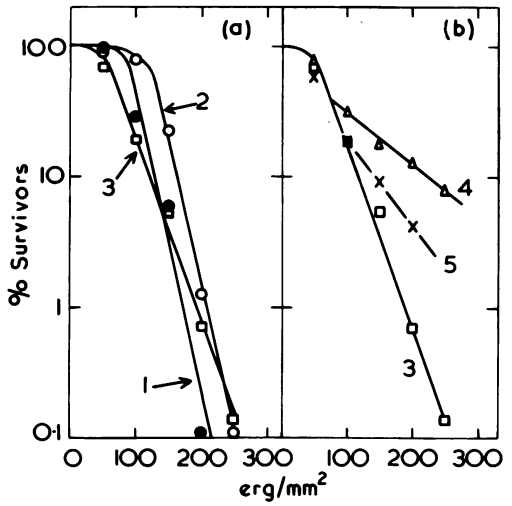

FIG. 2. *UV inactivation of exponential-phase and early sporulating B. cereus T. Fractions of exponential-phase and sporulating cultures were irradiated in dim light and immediately plated. Each sample was microscopically classified with respect to the number of cells per chain and the morphological stage of sporulation. The numbered curves correspond with the numbered samples in Table 1 in which the morphological parameters are listed. Curve 1, $t_{-0.6}$; curve 2, $t_{4.5}$; curve 3, $t_{7.75}$; curve 4, $t_{9.1}$; curve 5, $t_{8.2}$.*

of the next sample showed a shoulder reduction of over 50% and a greater D_{10} (exp) value (73 ergs/mm²) (Fig. 2a and b, curve 3; Table 1). A dramatic decrease in n from 800 to 6 was also observed. Thus, it is evident that a fundamental change in the UV resistance occurred upon progression from stage II to stage III. Note that in this population 86% of the chains contained stage III forespores. In addition, 43% of those chains also contained early stage IV forespores (Table 1). Thus, even though the population was heterogeneous, essentially monophasic inactivation kinetics (typical of homogeneous populations) were obtained. This suggests that forespores in stage III and early stage IV exhibit the same UV resistance.

Another change in UV resistance occurred in the next sample (Fig. 2b, curve 4; Table 1). A biphasic inactivation curve was obtained. This sample contained 55% of chains with stage III and early stage IV forespores, 34% with mid-stage IV forespores (Table 1) and 27% with RFS. The fraction of the population more resistant to UV doses from 80 to 250 ergs/mm² accounted for about 40% of the total and possessed a D_{10} (exp) of 212 ergs/mm². The other 60% exhibited a pattern of UV resistance similar to that of stage III forespores (curve 3). Since this sample contained 34% of chains with mid-stage IV forespores, it appeared that the mid-stage IV forespore might account for the 40% of the population that exhibited a D_{10} (exp) of 212 ergs/mm². This is further substantiated by comparison with a population from another experiment (Fig. 3) which is also plotted in Fig. 2b (curve 5). This population contained over 16% of chains with mid-stage IV forespores and less than 1% RFS. Note that this curve is also biphasic, with about 80% of the population being similar in UV resistance to stage III forespores. The remaining 20% exhibited a D_{10} (exp) of 132 ergs/mm². We suggest, therefore, that the appearance of mid-stage IV forespores confers upon that chain a D_{10} (exp) in excess of 130 ergs/mm².

RFS and free spores. UV resistance curves and the morphology and resistance parameters of a culture at stages of sporulation later than those of Fig. 2 are given in Fig. 3 and Table 2. The earliest sample (open circles, 0 h) is also curve 5, Fig. 2. In the sample 1 h later, the D_{10} (exp) had increased further, and the formation of another class of UV resistant cells was indicated by the appearance of a plateau that extended beyond 300 ergs/mm². About 16% of the population was of this high-resistance class. The percentage of chains with at least one RFS in this culture was about 18 (Table 2).

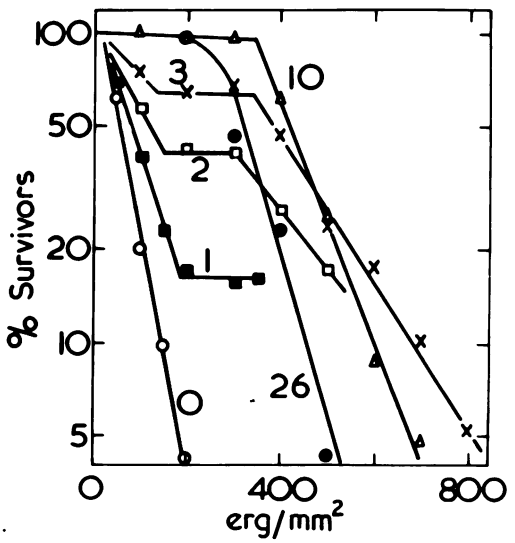

FIG. 3. *UV inactivation of sporulating B. cereus T. Fractions from a sporulating culture were removed at appropriate intervals, diluted, irradiated, and immediately plated. Morphological development (Table 2) and DPA accumulation (Fig. 4) were measured. Culture samples for UV inactivation were removed at zero ($t_{8.2}$, start of experiment), 1, 2, 3, 10 and 26 h. All spores were freed from the sporangia by 26 h.*

As sporulation progressed, the proportion of the high-resistance class with plateaux extending to 300 ergs/mm² increased (Table 2, Fig. 4). During sporulation (0 to 10 h) chain length remained about the same (Table 2). The D_{10} (exp) increased to a maximum at about 3 h and thereafter decreased progressively until sporulation was complete and all spores were free (Table 2, Fig. 4). Our earlier study (5) showed that DPA sensitized *B. cereus* spores to UV. The decline in UV resistance during sporulation reported here also appeared to be related to the accumulation of DPA by the forespores (Fig. 4).

The value of n when most of the cells (and thus all of the chains) contained an RFS (10-h sample, Fig. 3) was 19. This value divided by the average chain length gives an n value of 5.6 per forespore. The value for free spores was 20.

The temporal relationships between the development of (i) UV resistance (D_{10} [exp]), (ii) chains with at least one RFS, (iii) RFS as a percent of total cell units, (iv) high resistance class in the population and (v) DPA are shown in Fig. 4. The increase in numbers of chains with one or more RFS preceded the increase in high-resistance class by about 30 min. The most significant increase in UV resistance (D_{10} [exp]) began about the same time as the build-up in the high-resistance class population, and their rates of increase were similar. RFS development (percentage of total cell units) preceded DPA synthesis by about 30 min, and both increased at a similar rate (0.3%/min).

The D_{10}(exp) began to decrease steadily about 1 h before the proportion of the high-resistance class reached 80%. The decrease in D_{10} (exp) value was most rapid about midway in DPA synthesis (Fig. 4).

UV resistance of DPA⁻ mutant supplemented and unsupplemented with DPA. The UV resistance of the mutant culture is shown in Fig. 5 (a and b). The survivor curve at late stage II-early stage III is shown in both a and b for reference (open circles). At this point the culture was divided (DPA added to one part), and the development of UV resistance in each culture followed. The average chain length of each culture remained similar throughout (Table 3). The development of UV resistance of the DPA-supplemented culture (Fig. 5b) was similar to that of the wild type (Fig. 3). Unsupplemented cultures containing 2 and 69% of chains with RFS, however, exhibited very large plateaus. In this experiment the plateaus were greater than 1000 ergs/mm². In another experiment (data not shown) the plateau was about 1300 ergs/mm² (28% chains with RFS, average chain length 3.2). By the time all chains contained one or more RFS, the plateau had decreased to about 650 ergs/mm² (Fig. 5a).

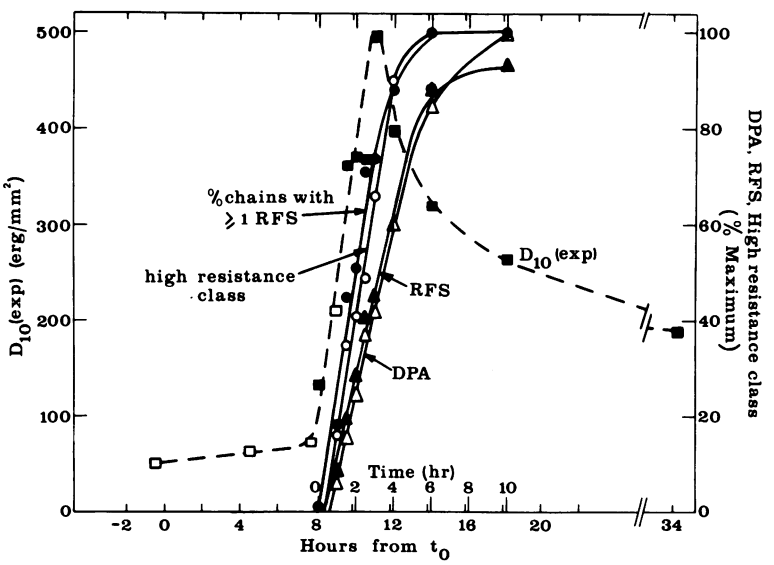

FIG. 4. *The temporal relationship between development of UV resistance (D_{10} exp), morphological changes, and DPA formation. Data from experiment 1 (Table 1) and 2 (Table 2) are plotted. D_{10} (exp) for experiment 1 (□) and for experiment 2 (■). The increase with time in the percentage of chains with at least one refractile forespore (●); in the high-resistance class (○); and in total cell units with an RFS (▲); and DPA content in the cells in the culture (△), are given. DPA content is expressed as a percent of maximum which was taken as the level (micrograms per milliliter) in the 10-h culture ($t_{18.2}$) and was, in fact, the same as in the free spores (55 μg/ml).*

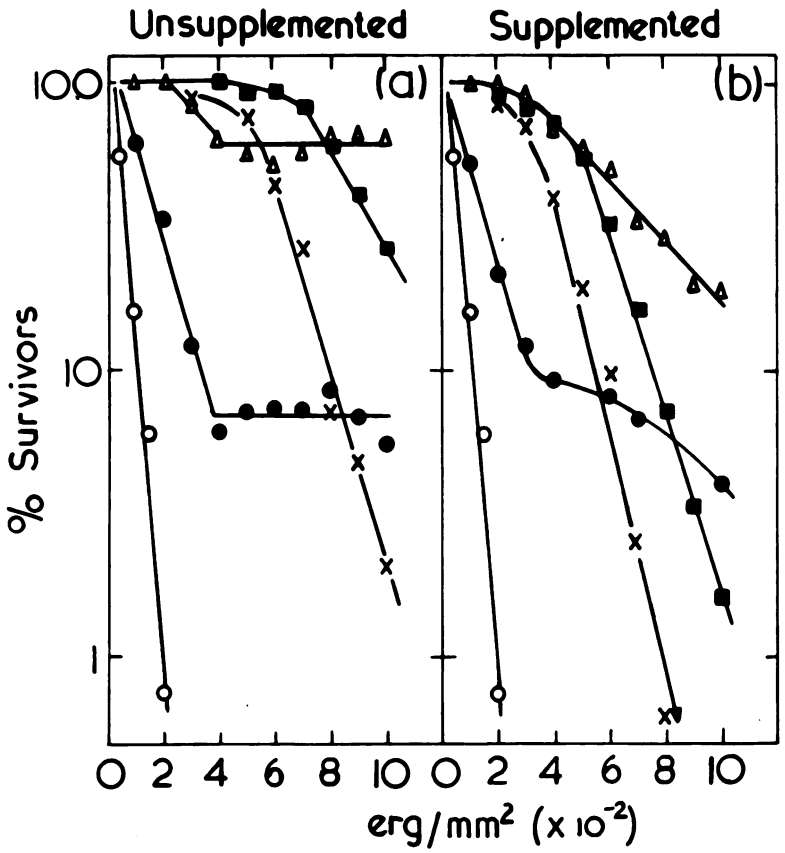

FIG. 5. *UV inactivation of sporulating DPA⁻ mutant of B. cereus T. A sporulating DPA⁻ mutant culture was divided (zero time) when the cells contained stage II forespores (O). One culture (Fig. 5b) was supplemented with DPA to give a final concentration of 100 μg/ml. The other culture was unsupplemented (Fig. 5a). Microscope data for the irradiated samples removed at 2.0 (●), 2.8 (Δ), and 3.7 (■) h are listed in Table 3. Free spores (sampled at 26 h after the culture was divided) were also irradiated (×). Free spores of unsupplemented and DPA-supplemented cultures contained 0.17 and 37.9 μg of DPA per ml, respectively.*

The free spores of the unsupplemented culture were more UV resistant than spores of the DPA-supplemented one (Fig. 5a and b, shoulders 500 and 300 ergs/mm², D_{10} [exp] of 310 and 240 ergs/mm², 0.17 and 38 μg of DPA per ml culture, respectively). Thus, the level of UV resistance was greater in the unsupplemented than in the DPA-supplemented culture both during sporulation and in the mature spore.

High-resistance class. The high-resistance class seen in the wild-type culture (Fig. 3) and in the mutant (Fig. 5) does not relate perfectly to the appearance of chains containing one or more RFS (Fig. 6) but rather lags behind it by 15 to 20%. At a rate of forespore development of about 0.4%/min, this represents a 25- to 30-min lag. In all our experiments, samples were irradiated and plated within 20 min. Similarly, microscope counts on undiluted culture samples (chilled in ice) were completed within 20 min. Thus, it is unlikely that technical procedures could account for the time difference between appearance of chains with RFS and the high-resistance class.

DISCUSSION

This study illustrates the relative complexity of the acquisition of UV resistance during sporulation. The acquisition can be divided into six categories (Fig. 7). First as a culture progresses from exponential growth to stage II of sporulation, the shoulder and D_{10} (exp) increase slightly. Since this change is already seen in pre-stage II cultures, we conclude that changes during spore septation and segregation of spore DNA do not confer a marked UV resistance.

The second category is represented by a decreased shoulder and n value, and an increased D_{10} (exp) (Fig. 2 and 7). These changes

occur as the number of stage III and early stage IV forespores increases. From evidence already discussed, it appears that stage III and early stage IV have similar UV resistance. Published evidence for *B. cereus* (3) and *B. subtilis* (4) suggest that mother cells at stage III are not able to divide. If the mother cell lost viability (ability to form a colony) upon forespore engulfment (stage III), one might expect (i) a change in D_{10} (exp) if the stage III forespore DNA were in an environment different from the stage II forespore DNA and (ii) a decreased shoulder if the target volume of the colony-forming unit decreased from that of the mother cell DNA plus stage II forespore DNA to only stage III forespore DNA.

The third category of UV resistance is typified by a marked increase in D_{10} (exp) (Fig. 2 and 7) to over 130 ergs/mm^2. Since populations exhibiting this higher D_{10} (exp) relate to the presence of mid-stage IV spore forms, we suggest this higher D_{10} (exp) value and greater UV resistance are actually conferred by mid-stage IV. The mechanism of the D_{10} (exp) increase is not readily identified. During this period, cortex synthesis begins, the forespore DNA migrates to the protoplast periphery, and the forespore cytoplasm takes on the appearance of the mature spore (electron-dense, featureless, ribosomes not discernible [9]). As a possible result of the above changes, the forespore DNA could assume the conformation in mature spores and thus be more resistant to lethal damage (2).

TABLE 3. *Chain length and refractile forespore development in DPA$^-$ mutant of B. cereus T*[a]

Hours[b]	No. of cells per chain		Percentage of chains with a refractile forespore	
	-DPA[c]	+DPA[c]	-DPA	+DPA
2.0	2.7	2.6	2	8
2.8	3.0	2.9	69	65
3.7	2.7	2.9	98	93

[a] Refer to Fig. 5 for inactivation curves.

[b] Hours from time culture was divided and DPA added.

[c] -DPA, Unsupplemented; +DPA, supplemented with 100 μg of DPA per ml. See text for details.

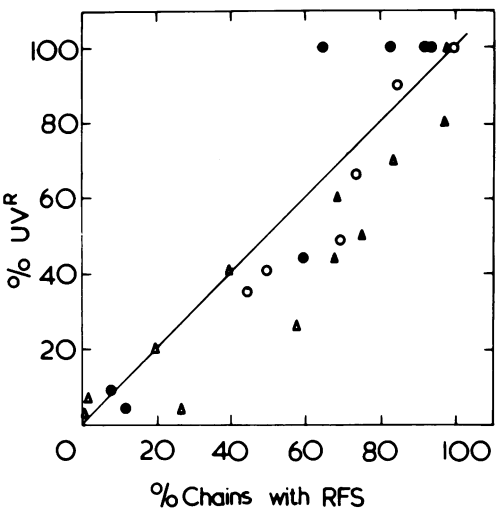

FIG. 6. *Relationship between chains with an RFS and high-resistance class. Data from a number of experiments have been combined. The proportion of the population of the high-resistance class (% UV^R, ordinate) is plotted against the proportion of chains with at least one RFS (abscissa). Wild-type (Δ), DPA-supplemented mutant (●), unsupplemented mutant, (○). Line drawn at a 45° angle for reference.*

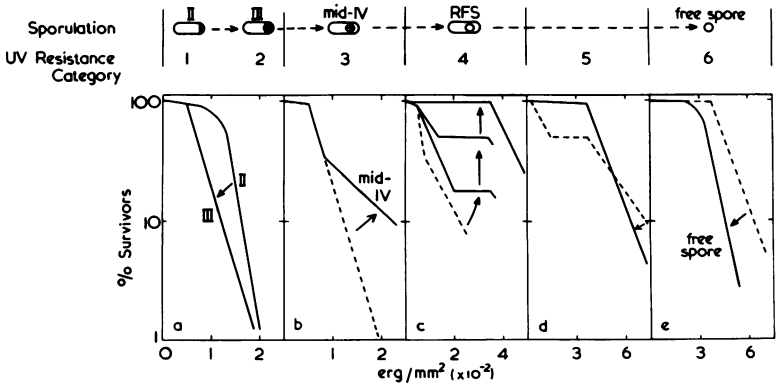

FIG. 7. *Categories of UV resistance during sporulation of B. cereus T. Categories of UV resistance during sporulation (solid lines) are shown with their associated morphological stages. For reference, the UV resistance pattern of the previous stage (broken line) has been superimposed for each category. See text for explanation of changes.*

This is supported by the observation with the unsupplemented DPA⁻ mutant in which case the D_{10} (exp) of mid-stage IV forespores and mature free spores was of the same order (about 300 ergs/mm², Fig. 5a).

The fourth and fifth categories of UV resistance concern two phases of the same survivor curve. The fourth is typified by the appearance of a plateau (Fig. 3, 7) which extends to over 300 ergs/mm². The proportion of this high-resistance class increases as sporulation progresses but lags behind RFS distribution among chains by about 30 min (Fig. 4 and 6). It appears after the start of synthesis of cystine-rich spore coats (1, 15). Conceivably, spore coat disulfides could act as a "buffer" system for radicals (14) and vitiate much potential inactivation as a result of "indirect" damage to the spore. The fifth class of UV resistance is characterized by the progressive decrease in D_{10} (exp). DPA sensitizes both wild-type and mutant *B. cereus* spores to UV (5). The accumulation of DPA in the refractile forespore protoplast thus might sensitize the forespore DNA to UV damage. This idea is supported by data with the mutant (Fig. 5), where the DPA-supplemented mutant culture was less UV resistant than the unsupplemented one. During sporulation the latter had plateaus exceeding 1,000 ergs/mm², whereas the supplemented culture at equivalent morphological stages had plateaus of 400 to 600 ergs/mm² (Fig. 5). Previously (5), we showed that DPA levels of 0.05 to 0.15% reduced the shoulder, and DPA above 0.15% decreased the D_{10} (exp) in mutant spores. In wild-type spores (4 to 13% DPA) the D_{10} (exp) decreased with increased DPA. Thus we attribute the extremely large plateaus in the unsupplemented mutant to the absence of DPA, and the smaller plateaus in the supplemented mutant and wild type to the incorporation of DPA into the forespore protoplast and its subsequent UV sensitization.

Even in the absence of DPA, however, the shoulder decreased late in sporulation from well over 1,000 ergs/mm² (69% of chains with RFS) to 500 to 600 ergs/mm² (95% of chains with RFS). We have no satisfactory explanation for this and can only note that it occurred at a late stage.

Since *B. cereus* sporulates in chains, one would expect a general increase in resistance as each chain accumulated more high-resistance class cells. Calculations show that the accumulation of more RFS per chain only affects the plateaux width and not the D_{10} (exp).

Finally the sixth category is represented by the free spore. Comparing the UV inactivation curves of free spores with those obtained when RFS were present in over 90% of the chains it can be seen that the D_{10} (exp) decreased accompanied by a 30 to 50% reduction in the shoulder. The n value increased to 20. This change in n value from 6 for forespores in chains (category 5) to 20 apparently results from spore maturation changes.

Although this study has been carried out with an organism which has the disadvantage of sporulating in chains, it has revealed several interesting features about the acquisition of UV resistance during sporulation. These experiments may prove more fruitful with a sporulation system which differentiates predominantly in single cells, e.g., a *Clostridium* system. This system has the advantage theoretically of allowing more precise data collection and interpretation in addition to the intriguing possibility of physically separating cells in different stages of sporulation by density gradient methods (15) and then characterizing the isolated populations with respect to morphology, biochemistry, and photoradiobiology.

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