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Dependence on Growth Phase of Ultraviolet Sensitivity in

Pyrimidine Dimer-Excision Mutants of Bacillus subtilis

CHARLES T. HADDEN AND DANIEL BILLEN

The University of Tennessee—Oak Ridge Graduate School of Biomedical Sciences,
and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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Send proof to: Dr. Charles T. Hadden
Biology Division
Oak Ridge National Laboratory
Post Office Box Y
Oak Ridge, TN 37830

We have compared the survival of Uvr^+ and Uvr^- strains of Bacillus subtilis after ultraviolet irradiation (UV), using two Uvr^- mutations, uvr-1 and uvs-42. In some genetic backgrounds, stationary-phase cultures of the Uvr^- mutants comprise two populations, one of which, the resistant fraction, appears to have about the same UV sensitivity as Uvr^+ strains. After starvation for thymine, the resistant fraction was larger, but chromosome alignment by amino acid starvation did not increase the resistant fraction above what it had been in stationary-phase cultures. In a different genetic background the same Uvr^- mutations did not show a marked UV-resistant population in stationary phase, implying that an alternative repair system or different regulation of repair of UV damage may operate in some strains.

It is generally accepted that the most significant target for killing bacteria by irradiation is deoxyribonucleic acid (DNA). Consequently, the ability of a cell to survive radiation damage depends to a large extent on its ability to repair DNA damage caused by radiation. Among the photo-products appearing in DNA following irradiation with ultraviolet light (UV), pyrimidine dimers appear to contribute most significantly to inactivation of cells (4,23,24,26,30).

One of the mechanisms of post-UV repair, as it is currently understood, involves enzymatic removal of pyrimidine dimers (4,5,24), synthesis of new DNA to fill the resulting gap (3,4,14,15), and closing of the break in the DNA strand (22,25). A number of bacterial mutants (Uvr^-) lack the ability to excise pyrimidine dimers (4,13,24,27,28). These mutants are extremely sensitive to UV.

When cells of Escherichia coli are starved for amino acids before irradiation, they become more resistant to UV (8,17,20). However, if DNA synthesis is inhibited at the same time, the ability to excise pyrimidine dimers decreases (21); thus we say the cells have become tolerant of pyrimidine dimers. We have found that amino acid-starved or stationary-phase cultures of some Uvr^- strains of B. subtilis are heterogeneous, with a population of phenotypically Uvr^+ cells. As in starved cells of E. coli, survival seems not to be related to excision of pyrimidine dimers, but, unlike the result with E. coli, the effect of growth stage or of starvation on UV sensitivity in Bacillus subtilis is more pronounced in Uvr^- strains than in Uvr^+ strains. Since the heterogeneity of sensitivity is absent in one isogenic set of strains, the sensitivity must depend on the genetic background.

MATERIAL AND METHODS

Bacterial strains. The strains used are listed in Table 1.

T-1

Media and growth of bacteria. Cells in liquid culture were grown at 37 C in modified (2) Spizizen's salts medium (SM) plus 0.5% glucose, 0.05% vitamin-free casamino acids (Difco), and 50 µg/ml of any required supplement (thymidine (dThd) in the case of Thy⁻ strains). A Klett-Summerson photoelectric colorimeter with a no. 42 filter was used to measure turbidity of cultures. Logarithmically growing cells were harvested at a Klett reading of 90 to 120.

Thymine and amino acid starvation experiments were carried out by harvesting cells by centrifugation, washing them twice with SM, and resuspending them in SM with added glucose, but with no other supplements (1). After the desired period of starvation, the viable titer of the culture was determined; cells were then processed for irradiation.

Irradiation. Cultures were harvested, washed, and prepared for irradiation as previously described (7). UV irradiation was carried out using 15-W germicidal lamps (General Electric), at a dose rate of 44.4 or 2.5 J/m² per min (7).

Survival of colony-forming units. Samples from cell suspensions were diluted in saline citrate buffer (SSC; 0.15 M NaCl, 0.015 M sodium citrate) and plated on nutrient agar (Difco) or nutrient agar plus 50 µg dThd/ml.

RESULTS

Sensitivity to UV. The relative sensitivities of some of the strains

used in this study have been reported (12,16), but the results of the two reports cannot be compared because different conditions were used. Reiter and Strauss (16) found that the D_{37} doses for FB13 (uvr-1) and FB8 (Uvr^+), growing in amino acid-supplemented medium, were about 6 and 19 J/m^2 , respectively. In contrast, Munakata and Ikeda (12) found D_{37} doses of 0.8 and 8.8 J/m^2 for the uvs-42 mutant and its parent, growing exponentially in tryptone-yeast extract broth. Because of the dissimilarity of these results, we have compared the mutants and their parents under identical conditions.

Figure 1 shows the colony-forming response to UV of cells growing exponentially in a glucose-salts medium supplemented with casamino acids. Under these conditions the two mutants show similar sensitivities, as do their parents (Table 2). The D_{37} doses observed for the mutants are slightly different; however, both strains of the FB8-FB13 (uvr-1) pair are slightly more resistant than the corresponding strains of the FB50 (uvs-42)-FB51 pair.

Both FB13 (uvr-1) and FB50 (uvs-42) show a high initial sensitivity, but at doses above 20 J/m^2 , the slope of the survival curve changes to approximately that of the parent strain. As shown below, this probably indicates that there are two populations of cells, a sensitive population, which is the major fraction of the culture, and a resistant population, which is able to survive more UV damage.

Effect of growth phase on UV sensitivity. In E. coli, the sensitivity of uvrA mutants grown in a glucose-salts medium supplemented with amino acids is not affected by growth phase (6,29). The same is true of B. subtilis (Fig. 2). Figure 2A shows the response to UV of FB13 (uvr-1)

F-1

T-2

F-2

and its Uvr^+ parent in both stationary and exponential phase, while Figure 2B shows the responses of FB50 (uvs-42) and its Uvr^+ parent. The initial slopes of the survival curves for the UV-sensitive mutants are independent of growth phase, while the Uvr^+ parents are more UV-sensitive in exponential phase than in stationary phase.

However, the proportion of UV-resistant cells in the Uvr^- cultures does vary with growth phase. Extrapolation from the more resistant fraction to zero dose shows that the resistant fraction of stationary-phase cells is about 3%. In contrast, tailing of the survival curve for exponentially growing cells is so gradual that no accurate determination of resistant fraction can be made. If the survivors of a UV dose of 25 J/m^2 to stationary-phase cells of FB13 (uvr-1) are regrown and their UV sensitivities tested under these same conditions, the results are the same as those of Figure 2 (data not shown). Thus the cells of the resistant fraction are not revertants but are physiologically different in their response to UV.

It is not likely that the rate of accumulation of damage in the cells is different in the two populations. For example, self-shielding can be ruled out as a cause for the UV-resistant component of stationary-phase cells since the dose-response curve does not change even if the cells are diluted tenfold before irradiation.

Influence of chromosome completion on size of the resistant fraction.
A possible explanation for enhanced UV resistance in the resistant fraction is that the sensitivity of a cell varies during its life cycle. Stationary-phase cultures contain few cells undergoing active DNA replication (31), so resistance to UV might be related to lack of replication,

and the chromosomes of the cells in the resistant fraction might be completed.

Starvation of thymine-requiring cells for thymine causes the death of actively replicating cells but not of cells with completed chromosomes (11). To determine whether resistant-fraction cells contain completed chromosomes, we subjected both stationary-phase and exponentially growing cells of FB50 (uvs-42) to thymine starvation for 9 h. Among the survivors of thymine starvation, the fraction of cells surviving 50 J/m² of UV was higher than in unstarved cultures, about 8-fold for stationary phase and 70-fold for exponentially growing cells (Table 3).

T-3

We interpreted this result as indicating that the resistant-fraction cells are more likely than the sensitive cells to have completed chromosomes. Accordingly, we predicted the converse, that by starving cells for amino acids and thus allowing completion but not reinitiation of chromosomes (1,11), we could increase the resistant fraction. Since most stationary-phase cells of the B. subtilis strains we used have uncompleted chromosomes (31), amino acid starvation should increase the resistant fraction above that found in stationary phase.

This prediction proved to be only partly correct in the following experiment. Exponentially growing cells of FB13 were washed free of amino acids and incubated in glucose-salts medium. At various times, the UV dose-response of the cells was determined. As shown in Figure 3, amino acid starvation for as little as 2 h caused the UV-resistant fraction to increase. Extrapolation to a UV dose of zero implies that by this time the resistant fraction is as great as in the stationary phase culture (Table 4). However, the slope of the curve for tryptophan-starved cells

F-3

T-4

implies that the resistance of the starved cells is not as great as that of stationary-phase cells. Furthermore, the size of the UV-resistant fraction does not increase upon further starvation for amino acids, although a 6-h starvation is sufficient to allow completion of rounds of DNA replication in almost all of the cells (1) and to confer resistance to thymine starvation on practically all the cells (data not shown). This shows that although a completed chromosome may be one property of a resistant-fraction cell, completion of chromosomes is not sufficient to cause UV resistance.

Induction of defective prophage. B. subtilis carries a defective prophage PBSX (18), which can be induced under various conditions, causing lysis of the induced cell. Induction by mitomycin C is more efficient in exponentially growing cells than in stationary-phase cells. It is possible then that the sensitive fractions of FB13 (uvr-1) and FB50 (uvs-42) consist of cells more susceptible to induction of PBSX by UV. In that case, a dose of 50 J/m^2 given to stationary-phase cells might be expected to cause lysis of the majority of the cells upon subsequent incubation in growth medium.

Cells of FB13 in both stationary and exponential phases were irradiated and then diluted into Penassay broth for further incubation. Figure 4 shows that neither stationary-phase nor exponentially grown cells treated with UV were lysed during subsequent incubation, although turbidity stopped increasing in exponentially grown cells about the time that lysis commenced in a mitomycin-C-induced culture. Therefore no conclusion can be drawn about differences in prophage induction in the UV-sensitive and UV-resistant fractions.

Effect of genetic background on resistant fraction. FB13 (uvr-1) and FB50 (uvs-42) come from different sources and are different in genetic background, but both have a large resistant fraction in stationary phase. An isogenic pair of UV-sensitive mutants, GSY1027 (uvr-1) (9) and FB56 (uvs-42) was made by transferring the UV-sensitivity markers into GSY1026 (Table 1). The dose responses of both UV-sensitive mutants are essentially the same in stationary phase as in exponential phase (Fig. 5), and starvation of GSY1027 for amino acids, as in the experiment of Fig. 3, has little effect on the UV dose response (Fig. 6).

F-5

F-6

DISCUSSION

The uvr-1 and uvs-42 mutations cause an approximately equal decrease in the ability of cells of B. subtilis to survive UV irradiation. These mutants have a decreased ability to excise pyrimidine dimers from their DNA (7,13,27,28), and it is presumably the persistence of these dimers that causes the death of the irradiated cells.

Cultures of some UV-sensitive mutants are phenotypically mixed, including a small fraction of cells with approximately the wild-type sensitivity to UV. In two genetic backgrounds, the resistant fraction is much larger in stationary-phase cultures than in rapidly growing ones, leading us to suggest that the resistant cells are in some undefined resting condition, during which they undergo certain processes absent in rapidly growing cells — processes which lead to the recovery of the cell. In another genetic background, however, the resistant fraction is not present in stationary phase, implying that either the recovery processes or the attainment of a physiological state in which these processes are undergone is genetically determined.

Kubitschek, et al. have shown (10) that there is no dependence of UV sensitivity in E. coli cells on stage of the cell cycle. However, the populations they studied responded homogenously to UV, unlike those of B. subtilis. Cells of the resistant fractions of FB13 (uvr-1) and FB50 (uvs-42) appeared more likely to be resistant also to thymineless death than the UV-sensitive cells, implying that they are in a state of arrested DNA replication, with completed chromosomes. However, a 2-h starvation of exponentially growing cells for required amino acids caused an increase in the UV-resistant fraction before rounds of replication could be completed (1). Furthermore, even more extensive amino acid starvation, which yields a higher proportion of cells with completed chromosomes than is found in stationary-phase cultures (1), did not increase the resistant fraction above the fraction in stationary-phase cultures. Thus completion of chromosomes per se is not sufficient to confer resistance to UV irradiation.

We were unable to show that lysis by induced prophages was the primary cause of cell death in either exponential or stationary-phase cells. Although exponentially growing FB13 (uvr-1) cells were lysed after a UV dose of 20 J/m^2 (data not shown), a dose of 50 J/m^2 did not cause cell lysis.

The ability of a significant fraction of stationary-phase cells of FB13 (uvr-1) and of FB50 (uvs-42) to survive UV damage remains unexplained. Arabinose-grown E. coli B/r Hcr⁻ cells can be made more resistant to UV by starvation for amino acids (17). However, in this case the entire population is affected, apparently homogeneously. In the B. subtilis mutants, the majority of the population is unaffected by starvation or by

growth phase (Fig. 2 and 3). After simultaneous starvation of E. coli B/r for amino acids and thymine, not only is UV survival enhanced (20) but pyrimidine dimer excision is depressed (21), revealing a tolerance for dimers in the starved cells. This tolerance depends on the Uvr⁺ genotype. We conclude that tolerance for pyrimidine dimers is induced in cells of B. subtilis FB13 (uvr-1) and FB50 (uvs-42) by non-growth conditions. We assume that some non-growth condition of the cells allows UV damage to be repaired or by-passed in these cells and that the condition is not present in derivatives of GSY1026, either because alternative repair systems are missing or because they are regulated in a different manner. The resistant state might be related to the induction of sporulation or of competence for genetic transformation, but we have no evidence along this line.

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TABLE 1. List of strains

Strain designation	Genotype	Source	Reference
FB8 (168)	<u>trpC2</u>	D. S. Nasser	
FB13 (168 Uvr ⁻)	<u>trpC2</u> , <u>uvr-1</u>	H. Reiter	17
FB50 (UVS)	<u>trpC2</u> , <u>met-14</u> , <u>sfr</u> , <u>thyA</u> , <u>thyB</u> , <u>uvs-42</u>	A. K. Ganesan	12
FB51 (UVR)	<u>trpC2</u> , <u>met-14</u> , <u>sfr</u> , <u>thyA</u> , <u>thyB</u>	A. K. Ganesan	12
GSY1026	<u>trpC2</u> , <u>metB4</u>	J. A. Hoch	9
GSY1027	<u>trpC2</u> , <u>metB4</u> , <u>uvr-1</u>	J. A. Hoch	9
FB56	<u>trpC2</u> , <u>metB4</u> , <u>sfr</u> , <u>uvs-42</u>	FB50 — x GSY1026 (transformation cross)	

TABLE 2. UV sensitivity of B. subtilis strains. The UV doses required to reduce survival to 37% of the initial level were taken from Fig. 1

Strain	D ₃₇ Dose (J/m ²)
FB8 (Uvr ⁺)	34.6
FB13 (<u>uvr-1</u>)	4.5
FB51 (Uvr ⁺)	25.4
FB50 (<u>uvs-42</u>)	2.9

TABLE 3. Survival of FB50 (uvs-42, thyAB) after thymine starvation^a

Growth phase	Time of starvation (h)	Relative CFU/ml	UV Survival (50 J/m ²) ^b	Normalized UV survival ^c
Stationary	0	1.00	8.2×10^{-3}	8.2×10^{-3}
	9	1.8×10^{-1}	6.2×10^{-2}	1.12×10^{-2}
Exponential	0	1.00	3.2×10^{-5}	3.2×10^{-5}
	9	4.3×10^{-4}	2.2×10^{-3}	9.5×10^{-7}

^a Details of the starvation procedure are given in Materials and Methods.

^b Ratio of cells surviving a UV dose of 50 J/m² to the viable titer before irradiation. The suspensions included many dead cells in the case of thymine starved cultures.

^c Ratio of cells surviving a UV dose of 50 J/m² to the viable titer before thymine starvation, calculated by multiplying column 3 by column 4.

TABLE 4. Size of the UV-resistant fraction of FB13 after amino acid starvation

Time of starvation (h)	% Resistant to UV ^a
0	0.03
2	1.0
6	1.1
10	1.1
Stationary phase	1.3

^a Intercept of a back-extrapolation of the data of Fig. 3 to 0 UV.

FIGURE LEGENDS

- Fig. 1. UV sensitivity of exponentially growing cells. Cells were grown and irradiated as described in Materials and Methods.
Symbols: o, FB8 (W.T.); c, FB13 (uvr-1); Δ, FB51 (W.T.);
Δ, FB50 (uvr-42).
- Fig. 2. Comparison of UV dose responses in stationary-phase and growing cells. Open symbols refer to stationary phase,
closed symbols to exponentially growing cells. Symbols:
(a) o, c, FB8 (W.T.); Δ, Δ, FB13 (uvr-1); (b) o, c, FB51 (W.T.);
Δ, Δ, FB50 (uvr-42).
- Fig. 3. Effect of amino acid starvation on UV dose-response in FB13.
Details are described in the text. Symbols: o, stationary;
c, exponential; Δ, starved for 2 h; Δ, starved for 6 h; □,
starved for 10 h.
- Fig. 4. Growth of FB13 in broth after UV. Cells were irradiated with
50 J/m², diluted 10-fold into Penassay broth, and subsequently
incubated at 37 C. Open symbols refer to stationary-phase cells,
closed symbols to exponentially growing cells. Symbols: o, c,
no UV; Δ, Δ, 50 J/m² of UV; c, induced with 0.4 μg mitomycin
C/ml broth.
- Fig. 5. UV dose response of GSY1026 derivatives. Growth and irradiation
were as described in Materials and Methods. Open symbols refer
to stationary-phase cells, closed symbols to exponentially
growing cells. Symbols: (a) o, c, GSY1026 (W.T.); □, □,
GSY1027 (uvr-1); (b) Δ, Δ, FB56 (uvr-42).

Fig. 6. Effect of amino acid starvation on UV dose-response in GSY1027.
Exponentially growing cells of GSY1027 were washed free of
amino acids as described above, and starved for an additional
8 h before the UV dose-response was determined. Symbols: o,
stationary phase; ø, exponentially growing cells; Δ, starved
cells.

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Fig. 1

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Fig. 2

30039

Fig 2

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Fig. 4

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Fig. 5

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Fig. 6