

Fate of Thymine-containing Dimers in the Deoxyribonucleic Acid of Ultraviolet-irradiated *Bacillus subtilis*

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The fate of ultraviolet-induced, thymine-containing dimers in the deoxyribonucleic acid (DNA) of *Bacillus subtilis* was investigated in both the wild type (UV^R) and an ultraviolet light-sensitive (UV^S) mutant. During incubation in the dark, dimers were excised from the DNA of the UV^R *B. subtilis*, but remained in the DNA of the UV^S mutant. About 40% of the excised dimers recovered in the wild type were in the acid-soluble fraction; the remainder were in the incubation medium. A UV^S mutant of *Escherichia coli* K-12, shown previously to be defective in dimer excision, was irradiated with ultraviolet light and incubated under visible light for 3 hr. About 65% of thymine-containing photoproducts were removed from the DNA. These photoproducts were not recovered in the acid-soluble fraction. In comparison, the UV^S mutant of *B. subtilis* lost only 13% of such photoproducts from DNA when exposed to light under the same conditions.

Irradiation of bacteria with ultraviolet light (UV) produces a variety of pyrimidine dimers in their deoxyribonucleic acid (DNA; 15). In *Escherichia coli*, there are two distinct mechanisms for repairing this damage; one operates in the presence of light (4), and one functions in the dark (20). The photoreactivating repair system is enzymatic (4, 10), requires light of wavelength 3,200 to 4,000 Å (8), and functions by splitting the pyrimidine dimers in situ (16, 21). It has been reported that *Bacillus subtilis* fails to undergo photoreactivation as measured by an increase in survival of UV-irradiated cells after exposure to visible light (A. Kelner, Radiation Res. Soc. Abstr., 1965, p. 35). Dark repair involves the excision of the intact dimers and is accompanied by degradation of the bacterial DNA (1, 2, 7, 14, 18).

A number of UV-sensitive mutants of *E. coli* have been isolated and found to be defective in their ability to excise dimers during incubation in the dark (2, 5, 7, 14). They were also found to be incapable of repairing UV-irradiated T1 phage (6, 10, 12).

Recently, a UV-sensitive mutant of *B. subtilis* was isolated which is also defective in repairing UV-irradiated phage (13). It was the purpose of

these experiments to determine the fate of UV-induced thymine-containing photoproducts in the DNA of both the normal and UV^S mutant of *B. subtilis* during post-UV incubation in the dark or under visible light. (UV^S and UV^R will be used to denote UV-sensitive and UV-resistant phenotypes; *uvr* will be used as the symbol for a genetic locus controlling pyrimidine dimer excision and UV sensitivity.) Experiments showed that thymine-containing dimers were excised in the dark from DNA of the normal strain but not from the UV^S mutant. Incubation in visible light had no significant effect on the number of dimers remaining in the DNA of the UV^S *B. subtilis* mutant. In contrast, a UV^S mutant of *E. coli* lost 65% of thymine-containing dimers from DNA when incubated in visible light after UV irradiation.

MATERIALS AND METHODS

Bacterial strains. A transformable strain of *B. subtilis*, JB1-49 (*ind⁻ his⁻ cys⁻ uvr⁺*), and a UV-sensitive mutant, JB1-49 23 (*ind⁺ his⁻ cys⁻ uvr⁻*), were kindly supplied by Bernard Strauss. The UV^S mutant was obtained by transformation of JB1-49, by use of DNA carrying a *uvr⁻* mutation as a donor (13). A growth requirement for low concentrations of thymine was introduced into both strains by the methods of Stacey and Simson (17). A thymine-requiring UV^S

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mutant of *E. coli* K-12 (AB2500) carrying *uvrA6*, and the UV^R strain AB2497, were also used (7).

Growth and labeling of cells. The minimal growth medium contained (per liter): (NH₄)₂SO₄, 2 g; sodium citrate, 1 g; MgSO₄, 0.2 g; glucose, 5 g; K₂HPO₄, 15 g; and KH₂PO₄, 6 g. The pH was adjusted to 7.2 with KOH. Cells were grown to late log phase in 6.0 ml of minimal medium supplemented with 0.6 ml of 5% Casamino Acids, 0.12 ml of 5% tryptophan, and 1.0 ml of methyl ³H-thymine (17 μg; specific activity, 14.5 c/mmole; New England Nuclear Corp., Boston, Mass.). The culture had a generation time of 50 min. Ten minutes before termination of growth, 100 μg of nonradioactive thymidine was added. The cells were washed two times in minimal medium by centrifugation and were then suspended in minimal medium for irradiation. More than 90% of the radioactivity was acid-precipitable in cells labeled in this manner.

Irradiation. Samples (30 ml) of minimal medium containing 4.5×10^7 cells per milliliter were irradiated in petri dishes (14 cm in diameter) on a shaking platform. The dose rate was 10 ergs per mm² per sec as determined by a General Electric Germicidal UV meter. The average dose to the cells was 1,600 ergs/mm² in all experiments. The survival of the resistant JBI-49 cells was 10⁻³; survival of the sensitive JBI-49 23 cells was 10⁻⁵.

Postirradiation treatment of cells. After irradiation, 100 μg of unlabeled thymidine was added to each petri dish. Control samples were immediately chilled in ice; the others were kept in the dark for 2 hr or in the light for 3 hr at 37 C. The latter were exposed to four fluorescent lights at a distance of 5 cm, with occasional stirring. They were then centrifuged and washed once with 1.5 ml of minimal medium, the wash being combined with the 30-ml incubation medium after centrifugation and saved for determination of radioactivity as described below. The packed cells were suspended in 1 ml of 5% trichloroacetic acid and kept at ice temperature for 45 min. Acid-soluble and acid-insoluble fractions were separated as previously described (2). They were dried under a stream of warm air, hydrolyzed at 175 C for 90 min with concentrated trifluoroacetic acid, and paper-chromatographed in butanol-acetic acid-water (200:30:75), following the procedure of Boyce and Howard-Flanders (2). Paper strips were dried in air and scanned for radioactivity.

Treatment of the incubation medium. The incubation medium was concentrated by evaporation to 7.5 ml and shaken with 10 mg of charcoal (19). Approximately 90% of the radioactive material was recovered from the charcoal after shaking for 2 hr with 10 ml of 10% pyridine in 50% ethyl alcohol. Samples of charcoal-eluted material were chromatographed without hydrolysis, as described for the acid-soluble and insoluble fractions. Radioactive material remaining near the origin was eluted, hydrolyzed with trifluoroacetic acid as described, and rechromatographed.

In a reconstruction experiment, one-half of the acid-soluble fraction of UV-irradiated, incubated, UV^R *E. coli* AB2497 was hydrolyzed and chromatographed as described. The other half was first adsorbed onto charcoal, eluted, hydrolyzed, and chromatographed. No difference in the ratio of radioactivity of

thymine-containing dimers to thymine was observed between the two samples, indicating that no selective loss of either dimers or thymine occurred upon adsorption and elution from charcoal.

RESULTS

Table 1 shows the numerical results of these experiments. Upon incubation, there was a decrease in photoproducts relative to thymine only in the UV^R JBI-49 strain. In the UV^S strain, JBI-49 23, no decrease in photoproducts relative to thymine was observed during incubation in the dark. Thus, as in *E. coli*, the UV^R strain of *B. subtilis* is able to remove preferentially photoproducts from the DNA during incubation in the dark, whereas the UV^S strain is incapable of doing so.

If the disappearance of photoproducts from the DNA occurred by excision as in *E. coli*, then the pyrimidine dimers should appear in the acid-soluble fraction after incubation. However, all of the radioactivity initially present in pyrimidine dimers could not be accounted for in the acid-soluble and -insoluble fractions, and it was found that approximately 60% of the excised photoproducts recovered after incubation appeared in the medium.

To test whether dimers still attached to phosphate were passing out of the cell, the incubation medium was adsorbed to and eluted from charcoal. Concentrated samples were chromatographed after elution in butanol-acetic acid-water without trifluoroacetic acid hydrolysis. Under these conditions, phosphorylated compounds remain close to the origin (2). It was found that over 90% of the radioactivity appeared as free thymidine or thymine, which are poorly separated in this system; the remaining activity was clustered in the region of the origin. The origin region was eluted, hydrolyzed, and rechromatographed. Three radioactive peaks were observed; two corresponded to the *R_F* values for the thymine-containing photoproducts, and the third corresponded to the value for thymine (2). The photoproducts were eluted, UV-irradiated in aqueous solution at a dose of 10⁵ ergs/mm², and rechromatographed. After this treatment, the radioactivity migrated at the same *R_F* as did thymine. These results indicate that the excision products containing the dimers remain phosphorylated in their passage through the cell into the medium.

The numerical results of the photoreactivation experiment can be seen in Table 2. Incubation in the dark had relatively little effect on the dimer-to-thymine ratios in acid-insoluble DNA obtained from the sensitive strains of either *B. subtilis* or *E. coli* K-12. However, exposure to

TABLE 1. Distribution of radioactivity incorporated as ³H-thymidine in cellular fractions of *Bacillus subtilis*

Strain	Treatment	³ H activity (counts/min)								
		Medium			Acid-soluble			Acid-insoluble		
		Photo-products ^a	Thymine	Ratio ^b	Photo-products ^a	Thymine	Ratio ^b	Photo-products ^a	Thymine	Ratio ^b
JB-1-49 (<i>uvr</i> ⁺)	UV; no incubation	<50	2,240	% <2.3	<50	10,720	% <0.47	2,840	423,000	% 0.67
	UV; incubated 37 C, 2 hr	1,030	147,250 ^c	0.70	648	4,500	14	675	270,000	0.25
	No UV; incubated 37 C, 2 hr	<50	7,200	<0.71	<50	4,140	<1.2	<100	407,000	<0.02
JB-1-49 23 (<i>uvr</i> ⁻)	UV; no incubation	<50	4,500	<1.2	<50	5,760	<0.87	5,910	581,000	1.1
	UV; incubated 37 C, 2 hr	<50	14,320	<0.35	<100	17,820	<0.56	5,340	592,000	0.90
	No UV; incubated 37 C, 2 hr	<50	8,400	<0.60	<100	15,570	<0.60	<100	639,000	<0.02

^a Sum of the radioactivity of thymine-thymine dimers and thymine-uracil dimers.

^b Radioactivity in photoproducts divided by the radioactivity in thymine, expressed as percentages.

^c Total activity of thymine in the medium, of which 2,250 counts/min was phosphate-bound and 145,000 was free and unphosphorylated. The total activity of the photoproducts (1,030 counts/min) was associated with the phosphate-bound fraction.

TABLE 2. Effect of light on UV-induced thymine-containing dimers in DNA of UV-sensitive strains of *Escherichia coli* (AB2500) and *Bacillus subtilis* (JB 1-49 23)

Strain	Treatment	Distribution of radioactivity (counts/min)					
		Acid-soluble			Acid-insoluble		
		Photo-products ^a	Thymine	Ratio ^b	Photo-products ^a	Thymine	Ratio ^b
<i>B. subtilis</i> JB 1-49 23 (<i>uvr</i> ⁻)	UV; no incubation	<50	2,140	% <2.3	5,490	653,000	% 0.84
	UV; dark incubation 37 C, 2 hr	315	12,400	2.6	4,000	507,000	0.79
	UV; light incubation 37 C, 3 hr	206	25,700	1.2	3,680	502,500	0.73
<i>E. coli</i> AB2500 (<i>uvr</i> ⁻)	UV; no incubation	<100	25,900	<0.37	6,920	860,000	0.80
	UV; dark incubation 37 C, 2 hr	117	5,130	2.3	4,260	716,500	0.62
	UV; light incubation 37 C, 3 hr	297	29,400	1.0	1,540	573,000	0.27

^a Sum of the radioactivity of thymine-thymine dimers and thymine-uracil dimers.

^b Radioactivity in photoproducts divided by the radioactivity in thymine, expressed as percentages.

visible light reduced the ratio in *E. coli* K-12 by 65% and in *B. subtilis* by 13%.

DISCUSSION

The results of these experiments indicate that the dark-repair mechanism in *B. subtilis* exhibits

properties similar to *E. coli* with regard to excision of UV-induced photoproducts. There is a preferential removal of thymine-containing dimers from the DNA upon incubation of the UV^R strain in the absence of light, whereas the UV^S strain is deficient in the excision mechanism.

The loss of photoproducts from DNA in strain JB1-49 (*uvr*⁺) during incubation is recorded in Table 1. About 75% of the photoproducts were released from DNA during incubation, but only 35% of the thymine radioactivity was solubilized during the same period. The photoproducts could be released by degradation of 75% of the DNA followed by 40% reincorporation of breakdown products (but not photoproducts). However, it seems unlikely that appreciable incorporation could occur after degradation of 75% of the DNA; it is much more likely that the photoproducts are excised, as suggested in *E. coli* and *Micrococcus radiodurans*. In addition, direct evidence for dimer excision has recently been obtained with a partially purified cell-free extract prepared from *Micrococcus lysodeikticus* (W. L. Carrier, and R. B. Setlow, *Biochim. Biophys. Acta*, *in press*).

One difference between *E. coli* and *B. subtilis* is the presence of phosphorylated photoproducts in the postirradiation incubation medium of *B. subtilis*. A similar finding has recently been reported for *M. radiodurans* (1).

Whereas in *E. coli* there is a decrease of almost 65% of thymine-containing dimers from DNA during exposure to light for 3 hr, the results in Table 2 show that, in *B. subtilis*, the amount of dimers in DNA fell by only 13%, which is probably within the limits of experimental error. Thus, at this dose of UV irradiation, *B. subtilis* shows no significant removal of dimers by photoreactivation. There was a decrease in the dimer-to-thymine ratio of 20% in the UV^S *E. coli* K-12 AB2500 strain upon incubation in the dark. This may be indicative of some slight excision occurring in this strain at the UV dose administered to the cells. The decrease in the ratio of dimer to thymine is significantly less than that observed for the UV^R strain of *E. coli* K-12 incubated after irradiation under the same conditions (2).

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