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Pyrimidine Dimer Excision in a *Bacillus subtilis* Uvr⁻ Mutant

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A technique which allows the measurement of small numbers of pyrimidine dimers in the deoxyribonucleic acid (DNA) of cells of *Bacillus subtilis* irradiated with ultraviolet light has been used to show that a strain mutant at the *uvr-1* locus is able to excise pyrimidine dimers. Excision repair in this strain was slow, but incision may not be rate limiting because single-strand breaks in DNA accumulate under some conditions. Excision repair probably accounted for a liquid-holding recovery previously reported to occur in this strain. Recombinational exchange of pyrimidine dimers into newly replicated DNA was readily detected in *uvr-1* cells, but this exchange did not account for more than a minor fraction of the dimers removed from parental DNA. Excision repair in the *uvr-1* strain was inhibited by a drug which complexes DNA polymerase III with DNA gaps. This inhibition may be limited to a number of sites equal to the number of DNA polymerase III molecules, and it is inferred that large gaps are produced by excision of dimers. Because the *uvr-1* mutation specifically interferes with excision of dimers at incision sites, it is concluded that the *uvr-1* gene product may be an exonuclease which is essential for efficient dimer excision.

Uvr⁻ bacteria are characteristically very sensitive to UV irradiation. It was recently demonstrated that the survival of UV-irradiated *uvr-1* cells of *Bacillus subtilis* on nutrient agar plates could be enhanced if the cells were first incubated in a glucose-salts medium lacking amino acids required for growth (16). For recovery in liquid medium, the cells had to be in stationary phase and had to be mutant at the *uvr-1* locus. This recovery also occurs if the cells are plated immediately after irradiation on an agar medium on which growth is slow. A speculative interpretation was that a defect in normal excision repair allowed detectable repair by a "different type of excision repair" (16), so that the cells were killed if growth resumed before about 3 h after irradiation.

Excision repair of damage to UV-irradiated DNA often requires enzymatic removal of pyrimidine dimers (4, 22, 32). Endonucleases which incise DNA containing pyrimidine dimers have been described (5, 8, 12, 34), but it is not clear which exonuclease completes the excision process. *Escherichia coli uvr* mutants are defective in excision of dimers (4, 21, 30, 32), but to date none of the known *uvr* mutants of *E. coli* has been shown to have a defective exonuclease specific for UV-irradiated DNA. Tests of strains deficient in exonucleases V, VI, or VII have indicated that none of these enzymes is uniquely required for dimer excision after low UV doses (9, 11, 29), although a mutant lacking all three exonucleases is severely limited in excision (25). Experiments presented in this and the accompanying paper (18) imply that the *uvr-1* locus in *B. subtilis* may code for an exonuclease required for dimer excision. Dimer excision occurs in the *uvr-1* strain, but, in contrast to Uvr⁺ cells, *uvr-1* cells of *B. subtilis* appear to leave large gaps at the excision sites.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used were GSY1026 (*trpC2 metB4*) (21), GSY1027 (*trpC2 metB4 uvr-1*) (21), and FB56 (*trpC2 metB4 sfr uvs-42*) (16).

Growth of bacteria. For most experiments, cells were grown at 37°C in a defined medium consisting of modified Spizizen salts (3), 0.5% glucose, 0.05% acid-hydrolyzed casein (Difco), and 50 μg each of tryptophan and methionine per ml. DNA was labeled with ^{3}H by inclusion of 30 μg of 2′-deoxyadenosine and 20 μCi of [*methyl*-^{3}H]thymidine (71 Ci/mmol, Schwarz/Mann) per ml.

Density-labeled cells were grown in a deuterium oxide medium containing Spizizen salts (2), 5 mg of deuterated sugar mixture, and 50 μg of deuterated amino acid mixture per ml (both from Merck and Co., Inc.), 50 μg each of tryptophan and methionine per ml, and 1 μCi of [^{14}C]uridine (204 mCi/mmol, Schwarz/Mann) per ml. The cells were harvested by centrifugation, washed with modified Spizizen salts plus 0.5% glucose, and then incubated at 37°C at the original concentration in the (nonradioactive) light medium described above for 30 min to chase pools of heavy

isotopes. The cells were then harvested for irradiation as previously described (16).

Irradiation and postirradiation incubation. Cells were harvested by centrifugation, washed once with ice-cold modified Spizizen salts plus 0.5% glucose, and resuspended at a concentration of 5×10^7 to 7×10^7/ml. They were irradiated with a 15 W germicidal lamp, usually at a fluence rate of 0.375 J/m^2 per s. The cells were then diluted twofold into medium containing twofold-concentrated supplements and incubated for the indicated times. For inhibition of DNA polymerase III, 6-(*p*-hydroxyphenylazo)-uracil (HPUra) was used at 0.3 mM. HPUra was generously provided by B. L. Langley, Imperial Chemical Industries, Ltd., Macclesfield, England.

Spheroplast assay for pyrimidine dimers. The method for detecting pyrimidine dimers in spheroplasts (C. T. Hadden, Microbios, in press) was modified from Ganesan's procedure for *E. coli* (13). Conditions for alkaline sucrose gradient centrifugation and the method for calculating molecular weights have been described previously (17).

RESULTS

UV-induced incision and excision repair in Uvr$^-$ strains. Most of the data below are presented in terms of frequencies of incision breaks and of dimers detected as sites for nicking by *Micrococcus* UV endonuclease (8, 33; R. B. Setlow, W. L. Carrier, and J. Stewart, Biophys. J. **15**:194a, 1975) or as enzyme-sensitive sites (ESS). Figure 1 gives an example of the distribution of DNA from the wild-type strain GSY1026 in sucrose gradients after irradiation with 2.5 J of UV per m^2 and incubation in growth medium for 30 or 60 min. The decrease in number of ESS in DNA during postirradiation incubation shows that (i) dimers have been excised and (ii) the excision sites have been fully repaired. The breaks in endonuclease-treated DNA result from (i) dimers remaining in the DNA at sites which have not been incised and (ii) sites at which incision has occurred but repair has not been completed. The latter also appear in samples which have not been treated with endonuclease. At these sites there may still be a pyrimidine dimer adjacent to (or very near) the incision site. By calculating from the molecular weight distributions the frequencies of single-strand breaks in the DNA (Table 1), it is possible to estimate both the fraction of dimers removed with complete repair and the total number of incisions made in the process of repairing the DNA.

The number of sites repaired is defined as the reduction in number of breaks in UV endonuclease-treated DNA during postirradiation incubation. This number may be an underestimate because it is assumed that the number of single-strand breaks made by the cell at sites other than pyrimidine dimers is negligible. That is, single-strand breaks at nondimer sites would be counted as unrepaired dimers. However, because steps in the excision-repair system are usually

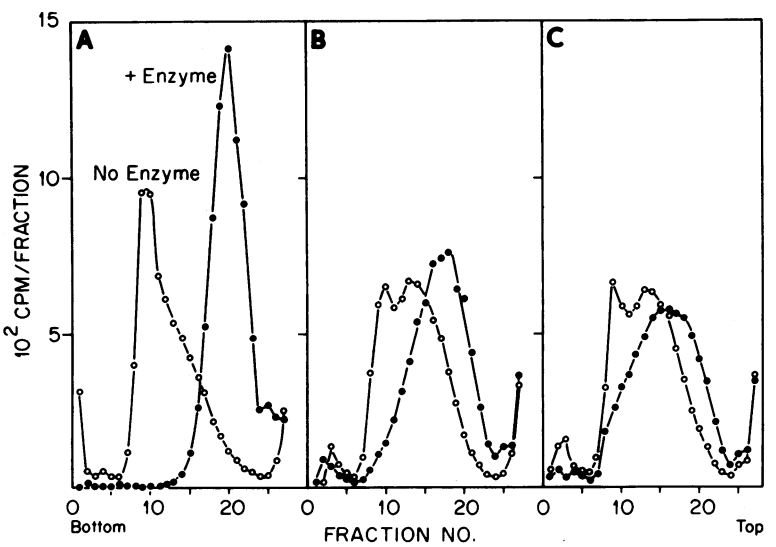

FIG. 1. *Alkaline sucrose gradient profiles of DNA from strain GSY1026. Stationary-phase cells labeled with [^{3}H]thymidine were irradiated with 2.5 J of UV per m^2 and incubated in growth medium at 37°C for the indicated times. They were then made into spheroplasts as described in the text, and part of each sample was treated with Micrococcus UV endonuclease. Conditions of centrifugation are described in the text.* ○, *No UV endonuclease;* ●, *plus UV endonuclease. (A) 0 min; (B) 30 min; (C) 60 min of incubation.*

TABLE 1. *Number on incision and repair events in GSY1026*[a]

Time (min)	UV fluence (J/m^2)	UV endonuclease	$(2/M_w) \times 10^9$	ESS per 10^9 daltons	Sites repaired	Unrepaired incisions	Total incisions
0	0	−	10.1				
	2.5	+	46.3	36.2			
30	2.5	−	11.7			1.6	
	2.5	+	20.2	8.5	26.1		27.7
60	2.5	−	11.8			1.7	
	2.5	+	15.8	4.0	30.5		32.2

[a] Data are from Fig. 1.

closely coordinated, the increase in number of single-strand breaks in samples without endonuclease treatment remains small (Table 1), so there cannot be many breaks at nondimer sites. These data are similar to those reported previously by a number of authors (1, 17, 19). Incision breaks disappear by either completion of excision repair or reversal of incision by ligase (30, 31). Reversal by ligase cannot readily be detected, but completion of excision repair is detectable by the disappearance of ESS. The total number of incisions can therefore be calculated by adding the number of new single-strand breaks to the number of sites completely repaired. These results are then graphed as fractions of the initial dimer sites (Fig. 2).

Figure 2 shows the kinetics of total incision and of completed repair in strains GSY1026 (Uvr^+), GSY1027 (*uvr-1*), and FB56 (*uvs-42*). Repair is rapid in the wild-type strain, and incision is only in slight excess over complete repair. In strain FB56 there is hardly any complete repair, although incision breaks accumulate slowly. This confirms the lack of dimer excision measured chromatographically (18). The *uvr-1* strain GSY1027 excises dimers slowly, but there is only a slight accumulation of unrepaired incision breaks. This implies in this strain either that the incision step may be rate limiting, with essentially normal completion of repair once incision has occurred, or that incision may be readily reversible, as appears to be true in *E. coli uvrC* strains (30, 31). This observation confirms results from chromatography experiments at higher UV doses (18).

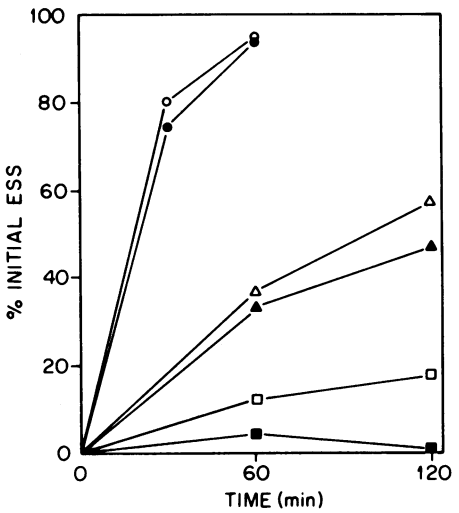

FIG. 2. *Kinetics of incision and repair of ESS. Stationary-phase cells of strains GSY1026 (Uvr^+), GSY1027 (uvr-1), and FB56 (uvs-42) were irradiated with 2.5 J of UV per m^2 and incubated in growth medium at 37°C for the indicated times before being made into spheroplasts and analyzed on alkaline sucrose gradients. Numbers of ESS repaired and total incisions were calculated as described in the text and Table 1.* ○, △, □, *Total incisions;* ●, ▲, ■, *ESS repaired.* ○, ●, *GSY1026;* △, ▲, *GSY1027;* □, ■, *FB56.*

Effect of growth conditions on repair in *uvr-1* cells. As noted previously (16), postirradiation recovery of *uvr-1* cells is prevented if the cells are incubated in nutrient broth or in the presence of caffeine, or if the cells are harvested for irradiation during exponential growth. Figure 3A shows that incubating irradiated stationary-phase *uvr-1* cells in nutrient broth markedly depresses repair of dimers. Incision is not reduced, so incision and completion of repair seem to be at least partially uncoupled. A similar inhibition has been obtained by incubating *uvr-1* cells in medium containing caffeine (C. T. Hadden and J. T. Lowry, Jr., unpublished data). Therefore, it appears that failure of recovery in stationary-phase *uvr-1* cells in nutrient broth is a result of inhibition of excision repair.

In contrast, incision and completion of repair are at least as rapid in exponentially growing cells as in stationary-phase cells in defined medium (Fig. 3B). Because exponentially growing *uvr-1* cells die despite being able to remove dimers from their DNA, it appears either that slow excision of dimers cannot ensure survival or that the dimers are being removed from parental DNA strands by recombination into

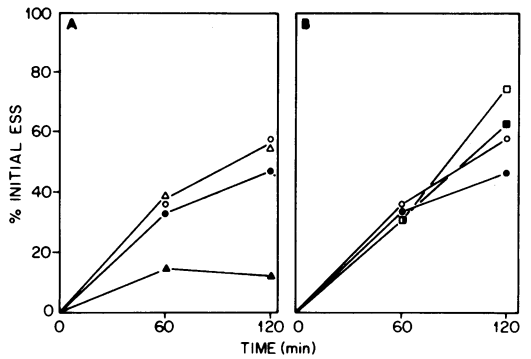

FIG. 3. *Effect of growth conditions on repair of ESS in GSY1027. Exponentially growing or stationary-phase cells of strain GSY1027 (uvr-1) were irradiated with 2.5 J of UV per m² and incubated at 37°C in either (A) Difco nutrient broth or (B) the amino acid-supplemented glucose-salts medium.* ○, △, □, *Total incisions;* ●, ▲, ■, *ESS repaired. Stationary-phase cells in* (○, ●) *defined medium or* (△, ▲) *nutrient broth;* □, ■, *log-phase cells in defined medium.*

newly replicated strands.

Exchange of dimers into newly replicated DNA. It was hypothesized previously (16) that postirradiation recovery involves recombination-dependent excision of pyrimidine dimers because recovery was reduced in *recA uvr-1* mutants. Although it was subsequently shown that the *recA* mutation affects metabolism of repaired DNA more than recombination (17), the possibility still remained that the disappearance of dimers was due solely to postreplicative repair (27, 28) by exchange of dimers from parental strands into newly replicated strands, as has been shown in an *E. coli uvrB* mutant (14). However, the experiments described below showed that in stationary-phase *uvr-1* cells, there was very little exchange of dimers, probably because there was very little DNA replication immediately after irradiation.

Because the newly replicated DNA had no radioactive label in the experiments described above, dimers exchanged into newly replicated strands would become undetectable without being removed from the cell. For recombination of dimers into daughter DNA to be measured, it is necessary to know both the frequency of dimers in the daughter DNA and the amount of daughter DNA. The frequency of dimers can be determined by putting a radioactive label ([^{3}H]thymidine) in the postirradiation medium and assaying ESS in the labeled DNA after various times of incubation. The amount of daughter DNA can be estimated by growing the cells in a deuterium-based medium (with a ^{14}C label as well) before irradiation and in a light medium afterward and determining by CsCl density gradient centrifugation the fraction of DNA replicated.

Figure 4 gives the sucrose gradient profiles of DNA from strain GSY1027 in such an experiment. As in Fig. 1, the parental DNA without enzyme treatment was smaller after 60 min than at 0 min, showing that some unrepaired incisions had occurred. The samples treated with UV endonuclease show that some repair of ESS had also occurred by 60 min postirradiation. Figure 4C shows that although some of the daughter strands of DNA were very small, there was a considerable amount of daughter DNA that was fairly large, with a few molecules as large as the unirradiated parental DNA. This larger DNA was extensively nicked by UV endonuclease, so most of the large daughter DNA was associated with DNA which contained dimers. Because incubation in nonradioactive medium after irradiation but before labeling does not change the sedimentation profiles (L. Dodson and C. T. Hadden, unpublished data), the large daughter strands are not merely added to preexisting dimer-containing DNA. Therefore, the dimers must have come from the parental DNA by recombination. Results are tabulated in Table 2. Figure 5 gives the kinetics of incision of parental DNA, repair of parental DNA, exchange of dimers into daughter DNA, and total excision repair, corrected for exchange of dimers out of parental DNA. Although it is significant that exchange of dimers into daughter DNA occurs, exchange does not account for the removal of more than a small fraction of dimers from parental DNA; most dimers must be removed by excision repair. More extensive exchange of dimers occurs in exponentially growing cells, and in *uvr-1* cells dimer excision takes place both before and after recombinational exchange (L. Dodson, M.S. thesis, University of Tennessee, Knoxville, 1979).

Formation of gaps at excision sites. HPUra is a specific inhibitor of replicative DNA synthesis (6, 7) which acts by inhibiting both polymerase and exonuclease activities of DNA polymerase III (24). In experiments in which repair synthesis was measured (18), it was assumed that DNA polymerase III plays, at most, an insignificant part in excision repair. When polymerase III was inhibited by HPUra and repair was measured by the UV endonuclease assay, however, the surprising result was that after low UV doses incision occurred in strain GSY1027, but completion of repair was almost completely blocked by HPUra (Fig. 6B). This was not true for the wild-type strain GSY1026 (Fig. 6A), so it initially appeared that polymer-

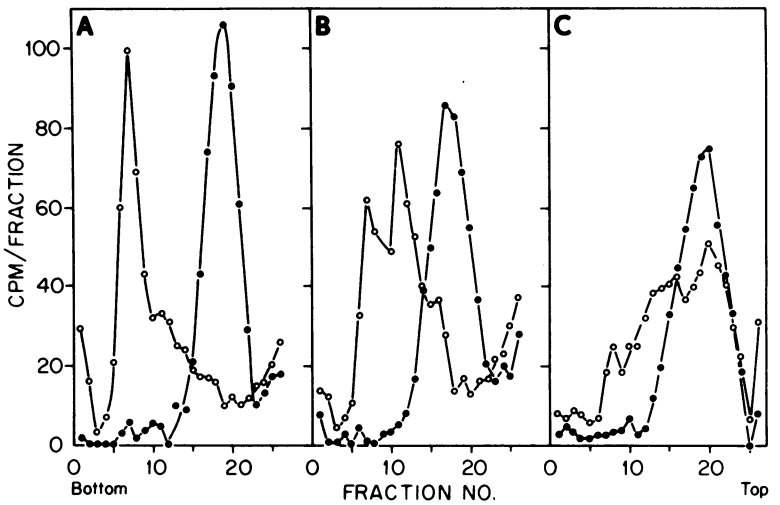

FIG. 4. *Alkaline sucrose gradient profiles of parental and daughter DNA from strain GSY1027. Stationary-phase cells of strain GSY1027 (uvr-1) grown in deuterium oxide-based medium and labeled with [^{14}C]uridine were irradiated with 2.5 J of UV per m^2 and incubated at 37°C in light medium containing [^{3}H]thymidine. Samples were made into spheroplasts, and a portion of each sample was treated with UV endonuclease. ○, No UV endonuclease; ●, plus UV endonuclease. (A) 0 min, parental [^{14}C]DNA; (B) 60 min, parental [^{14}C]DNA; (C) 60 min, daughter [^{3}H]DNA.*

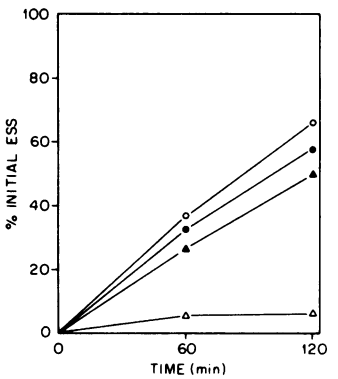

FIG. 5. *Repair and exchange of ESS in strain GSY1027. Data are taken from Fig. 4 and Table 2. ○, Total incisions in parental DNA strands; ●, ESS removed from parental DNA; △, ESS exchanged into newly replicated DNA; ▲, net removal of ESS.*

ase III was essential for the residual repair in *uvr-1* cells. However, the relatively high levels of repair synthesis and dimer excision (18) and recovery from sensitivity to nutrient agar (16) by GSY1027 in the presence of HPUra were not compatible with that conclusion.

The mechanism of inhibition by HPUra is to bind at the substrate site of polymerase III, making a tight complex comprising enzyme, inhibitor, primer, and template (24). This is possible only when polymerase III associates with gapped DNA. The inhibition of repair by HPUra is probably a result of the inhibited polymerase III complex blocking the excision site, as shown by the following experiments with higher UV fluences. Because the number of polymerase III molecules is low in stationary-phase cells (perhaps on the order of 30 to 50 molecules per cell) (10, 15), it should be possible to titrate the enzyme with excision gaps in the presence of HPUra. Additional gaps would then be free to be repaired. This was indeed the case when repair in the presence of HPUra was compared after fluences of 2.5 and 7.5 J/m^2. Figure 7 shows that after 3 h of incubation of cells irradiated by a fluence of 2.5 J/m^2 almost no repair had occurred in the presence of HPUra, although there were about 10 unrepaired incisions per 10^9 daltons of DNA (or probably about 30/cell). After 7.5 J/m^2, there was a significant amount of repair despite the drug, and again about 10 unrepaired incisions per 10^9 daltons of DNA. However, at both fluences the total number of incisions was only about 1/3 as great in the presence of the inhibitor, implying that polymerase III may still play some role in the excision process.

DISCUSSION

By use of an extremely sensitive enzymatic assay to detect pyrimidine dimers, it has been possible to show that a Uvr⁻ strain of *B. subtilis*, GSY1027, can excise dimers after low UV fluences. In 2 to 3 h this strain can repair 60% or more of the damage done by a UV fluence of 2.5 J/m^2. In contrast, another Uvr⁻ strain, FB56

TABLE 2. *Exchange of pyrimidine dimers into daughter DNA in GSY1027*[a]

Time (min)	UV endonuclease	Parental [^{14}C]DNA		Daughter [^{3}H]DNA		Percent ^{14}C in hybrid peak (5)	ESS in [^{3}H]-DNA per 10^9 [^{14}C]DNA[b] (6)	ESS per 10^9 daltons removed from [^{14}C]DNA[c] (7)	Total incisions in [^{14}C]DNA[d] (8)	Net ESS per 10^9 daltons repaired[e]
		$(2/M_w) \times 10^9$ (1)	ESS per 10^9 daltons (2)	$(2/M_w) \times 10^9$ (3)	ESS per 10^9 daltons (4)					
0	−	8.3	−	−	−	0				
	+	46.6	38.3	−	−					
60	−	10.0	−	17.9	−	10.7	2.2	12.4	14.1	10.2
	+	34.2	24.2	38.0	20.1					
120	−	11.7	−	16.4	−	32.3	2.2	22.0	25.4	18.8
	+	24.6	12.9	23.3	6.9					

[a] Cells of GSY1027 were grown in deuterium oxide-based medium with [^{14}C]uridine as described in the text. After irradiation with 2.5 J of UV per m^2, they were incubated in light medium containing [^{3}H]thymidine for the indicated times. They were then harvested for determination of ESS and for isopycnic centrifugation in CsCl (3). The frequencies of single-strand breaks and ESS in both parental and daughter DNA strands were calculated; then the number of dimers in daughter strands was calculated by multiplying the number of dimers per 10^9 daltons of [^{3}H]DNA that was replicated, giving the number of dimers in [^{3}H]DNA per 10^9 daltons of [^{14}C]DNA.

[b] (Column 4 × column 5)/100.

[c] ESS at 0 min − ESS at indicated time (column 2).

[d] Increase in column 1 (for −UV endonuclease data) + column 7.

[e] Column 7 − column 6.

(*uvs-42*), is unable to repair after even this low fluence.

The conditions for DNA repair in strain GSY1027 appear to be the same as those for recovery of ability to form colonies on nutrient agar (16), implying that the two processes are the same. There is, however, one major exception: although recovery of colony-forming ability does not occur in log-phase cells, repair of dimers does. Apparently by the time excision repair is completed under these conditions, some lethal event, perhaps related to replication of dimer-containing DNA, has occurred.

The nature of the *uvr-1* lesion in strain GSY1027 is not clear, but several possibilities might be considered. (i) There might be a defect in UV endonuclease. This seems unlikely because repair in the presence of HPUra or nutrient broth does not appear to be rate limited by incision. Furthermore, a defect in UV endonuclease would not account for the apparent formation of large gaps at excision sites specifically in the *uvr-1* mutant (Fig. 6; reference 18). (ii) There might be a defect in excision by DNA polymerase I-associated exonuclease (11). This is unlikely because the *polA* and *uvr-1* mutations map in different regions (15, 20, 23). Unless the *uvr-1* gene product is somehow required for activity of polymerase-associated exonuclease as hypothesized for *E. coli recL* (26), it is unlikely that DNA polymerase I is responsible for a significant portion of dimer excision in Uvr$^+$ *B. subtilis*. The observation of Chase and Masker (9) that dimer excision is essentially normal after low fluences in *polA*(Ex) mutants of *E. coli* tends to support the view that the *uvr-1* mutation is not like the *polA*(Ex) mutation. (iii) There might be a defect in a DNA binding or structural protein. In *E. coli uvrC* mutants there appears to be a defect in an activity which normally prevents DNA ligase from restoring incised strands before excision occurs, possibly by binding to the incised DNA (30) or by forming a complex which is essential for efficient dimer excision (31). In a similar way, a defective DNA-binding protein or structural component of an endonuclease complex in *B. subtilis* GSY1027 might prevent excision. Again, however, this explanation makes it difficult to account for the role of HPUra in blocking repair at low fluence. (iv) There might be a defect in UV exonuclease. This seems to be the simplest explanation for the results presented above. My speculative interpretation is that the major portion (but not necessarily all) of excision in Uvr$^+$ cells after a low UV dose is done by a specific exonuclease coded for by the *uvr-1* gene. In strain GSY1027 this enzyme is absent. Incision occurs but either

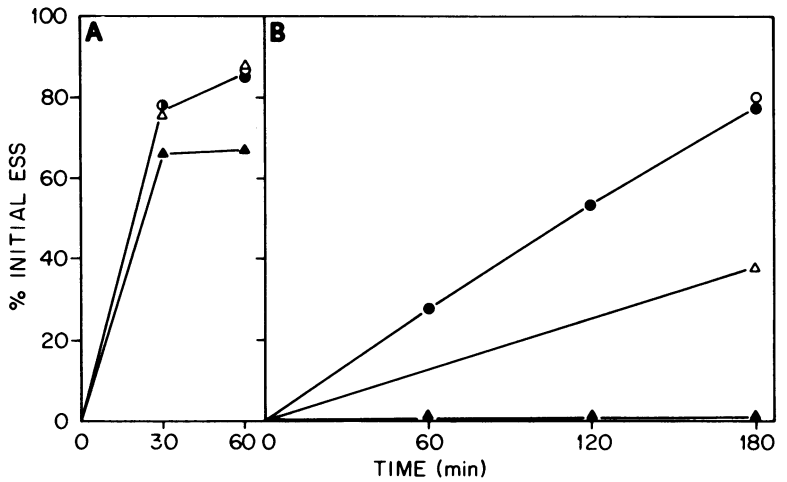

FIG. 6. *Inhibition of repair of ESS in strain GSY1027 by HPUra. Stationary-phase cells of strain GSY1026 (Uvr⁺) and GSY1027 (uvr-1) were irradiated with 2.5 J of UV per m² and incubated at 37°C in growth medium with or without 0.3 mM HPUra.* ○, △, *Total incisions;* ●, ▲, *ESS repaired.* ○, ●, *No HPUra;* △, ▲, *0.3 mM HPUra. (A) GSY1026; (B) GSY1027.*

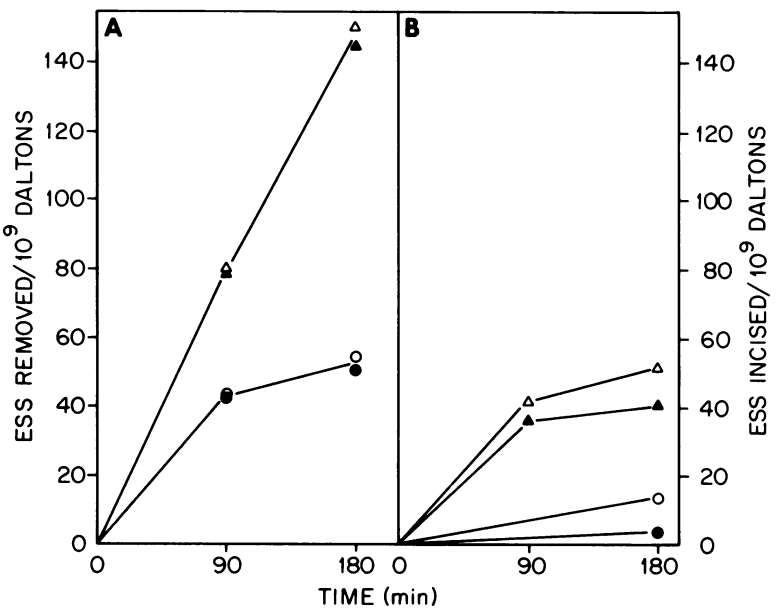

FIG. 7. *Effect of UV fluence on inhibition of repair in strain GSY1027 by HPUra. Stationary-phase cells of strain GSY1027 (uvr-1) were irradiated with 2.5 or 7.5 J of UV per m² and incubated in growth medium with or without 0.3 mM HPUra for the indicated times.* ○, △, *Total incisions;* ●, ▲, *ESS repaired.* ○, ●, *2.5 J/m²;* △, ▲, *7.5 J/m². (A) No HPUra; (B) 0.3 mM HPUra.*

is reversed by ligase or (infrequently) provides sites by strand displacement or excision of dimer-containing segments of DNA long enough to allow DNA polymerase III to bind at the excision sites. Inhibition of repair by the polymerase III-HPUra complex indirectly implies the existence of single-strand gaps in the DNA, because polymerase III is only inhibited by HPUra when it is associated with such a structure (24). This would explain the accumulation of single-strand breaks in the DNA during postirradiation incubation in the presence of HPUra.

Excision of dimers in oligonucleotides might be done by an endonuclease, or a single-strand-

specific exonuclease might remove dimers by degrading the displaced strand. It is clear that the excised fragment must be degraded at some point because dimers become acid soluble (18). The comparatively great inhibition of dimer excision in the *uvr-1* mutant is in marked contrast to the apparent ability of exonucleases to substitute for one another in *E. coli* (9, 11). The possibility that the *uvr-1* gene product is the sole enzyme used for normal dimer excision in Uvr^+ cells makes *B. subtilis* an attractive organism for isolation of a specific UV exonuclease.

ACKNOWLEDGMENTS

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