Transitory Germinative Excision Repair in *Bacillus subtilis*

**TZU-CHIEN VAN WANG** and **CLAUD S. RUPERT**

*Programs in Biology, The University of Texas at Dallas, Richardson, Texas 75080*

Received for publication 30 September 1976

*Bacillus subtilis* strains UVSSP-42-1 (*hcr<sub>42</sub> ssp<sub>1</sub>*) and UVSSP-1-1 (*hcr<sub>1</sub> ssp<sub>1</sub>*) are ultraviolet (UV) radiation sensitive both as dormant spores and as vegetative cells. These strains are unable to excise cyclobutane-type dimers from the deoxyribonucleic acid (DNA) of irradiated vegetative cells and fail to remove spore photoproducit from the DNA of irradiated spores either by excision (controlled by gene *hcr*) or by spore repair (controlled by gene *ssp*). When irradiated soon after spore germination, these strains excise dimers, but not spore photoproducit, from their DNA. This process, termed germinative excision repair, functions only transiently in the germination phase and is responsible for the high UV resistance of germinated spores and for their temporary capacity to host cell reactivation irradiated phages infecting them. The *recA* mutation confers higher UV sensitivity to the germinated spores, but does not interfere with dimer removal by germinative excision repair.

Spores of *Bacillus* acquire high ultraviolet (UV) radiation resistance during the process of germination (10, 11, 23, 24, 27). This transitory resistance to UV usually lasts for a relatively short time (minutes) in wild-type cells and has been suggested by Stafford and Donnellan (23) to be correlated with a decrease in the production of thymine-containing photoproducats during that particular period. Munakata recently showed that a *Bacillus subtilis* strain having UV-sensitive spores becomes much more UV resistant after spore germination than either the spores irradiated before germination or the vegetative cells and that this resistance lasts for hours when outgrowth of the cells is prevented (12). We have investigated this strain further, as well as related mutants, and find the high resistance stems from an additional repair mechanism functioning independently of the vegetative cell repair processes during the germination phase.

**MATERIALS AND METHODS**

Strains. The original strain (UVR) used in this work was *B. subtilis* Marburg 168 thy<sup>−</sup>try<sup>−</sup> from Parmer and Rothman (3). Isolation and construction of the strains SSP-1 (*ssp<sub>1</sub>*), UVSS-42 (*hcr<sub>42</sub>*), UVSSP-42-1 (*hcr<sub>42</sub>* *ssp<sub>1</sub>*), 25D4 (*hcr<sub>42</sub>* *recA*), 13 (*ssp<sub>1</sub>* *recA*), and G1 (*hcr<sub>42</sub>* *ssp<sub>1</sub>* *recA*) by Munakata et al. were described elsewhere (15, 17). The strains UVSS-1 (*hcr<sup>−</sup>*) and UVSSP-1-1 (*hcr<sup>−</sup>* *ssp<sub>1</sub>*<sup>−</sup>*) were constructed by introducing the *hcr* mutation from strain UVS-1 originally isolated by Munakata and Ikeda (14) into the strains UVR and SSP-1 by transformation. The mutations *hcr* and *hcr<sub>42</sub>* each render cells UV sensitive, defective in host cell reactivation (HCR), and unable to excise cyclobutane-type dimers in the vegetative phase. They also prevent the excision of spore photoproducit from irradiated spores upon germination (16). The mutation *hcr*, differs from *hcr<sub>42</sub>* in that the vegetative cells of *hcr<sub>42</sub>* produce single-stranded nicks in the deoxyribonucleic acid (DNA) after UV irradiation (as detected by alkaline sucrose gradient sedimentation), whereas cell of *hcr*, do not (Wang, unpublished data). The mutation *ssp* blocks the spore repair mechanism (15) which destroys spore photoproducit in situ (2). Spores of the double mutants *hcr*, *ssp*, or *hcr<sub>42</sub>* *ssp* are unable to remove spore photoproducit by any means, and are consequently UV sensitive (15). The mutation *recA*, obtained from J. Hoch (7), produces UV-sensitive, recombination-defective cells.

Media. Schaeffer nutrient broth medium was used to produce spores (25). Growth media used were: (i) TYB, which contains 10 g of tryptophane (Difco), 2.5 g of yeast extract (Difco), and 2.5 g of NaCl in 1 liter of water at pH 7.4; (ii) BHI, which contains 37 g of brain heart infusion (Difco) in 1 liter of water; and (iii) Spizizen minimal medium (22) supplemented with 10 μg of thymidine per ml, 0.02% Casamino Acids (Difco), and 50 μg of required amino acids per ml. Germination medium contains 100 μg of L-alanine per ml and 4 mg of glucose per ml in 0.1 M potassium phosphate buffer, pH 7.2.

Preparation of spores. Production of spores in Schaeffer nutrient broth has been described elsewhere (16). Spores were purified by centrifugation through a linear (50 to 100%) Renografin-76 gradient (Squibb and Sons, Princeton, N.J.) (13).

Irradiation. The low-pressure mercury-arc source for UV (254 nm) and measurements of its fluence rate have been described (15). *methyl-<sup>3</sup>H*thymine-
labeled spores were germinated without prior heat activation in germination medium at 37°C. At various times after germination, the cultures were placed in an ice bath, centrifuged, washed once with cold 0.1 M phosphate buffer, and suspended in the same buffer. A small portion of the cell suspension was irradiated at 4°C to determine cell survival by plating as previously described (15). The remaining portion was irradiated with a fixed fluence of 720 J/m² for determination of photoproduct formation. Fluence rates used were either 0.72, 2, or 6 W/m².

Assay for photoproducts. For determination of photoproduct formation in spores, the irradiated cells were placed in cold trichloroacetic acid at a final concentration of 5%, and the trichloroacetic acid-insoluble fractions were processed as previously described (16). For determination of photoproduct removal, spores were germinated for 10 min at 37°C in germination medium, centrifuged and washed with 0.1 M cold phosphate buffer, irradiated with 720 J/m², and suspended in the same buffer containing 1 mg of glucose per ml at about 2 × 10⁶ cells per ml. After incubation at 37°C for various times, cultures were removed, and trichloroacetic acid was added to a final concentration of 5%. Both trichloroacetic acid-insoluble and trichloroacetic acid-soluble fractions were processed for assay of photoproducts as previously described (16).

Host cell reactivation. Spores were suspended in BHI to about 5 × 10⁷ per ml, incubated at 37°C for various times, and then exposed to the virulent B. subtilis phage M2 (9) at a multiplicity of infection of 0.1 for 10 min. Cells were centrifuged, washed once with BHI, and plated in soft agar on prepared nutrient agar plates using log-phase vegetative cells (ca. 10⁸ per plate) as the indicator bacteria.

RESULTS

Photoproduct formation and cell sensitivities to UV. UV induces 5-thymyl-5,6-dihydrothymine [spore photoproduct, abbreviated Thy(α-5)HThy, or TDHT] as the major detectable thymine-containing photoproduct in the DNA of bacterial spores (1, 26). After germination, the nature of the photoproduct formed changes very rapidly from TDHT to predominantly cyclobutane-type dimers (23). We have followed photoproduct formation during spore germination in the UV-sensitive B. subtilis strain UVSSP-42-1 (hcr₄₂ ssp.) and G1 (hcr₄₂ ssp., recA1) in different growth phases. Strains UVSSP-42-1 and UVSSP-1-1 are comparable in their responses to UV. As expected from the mutations they carry (see Materials and Methods), both are UV sensitive as vegetative cells and as spores, yet they are UV resistant in their germination phase. Strain G1, carrying the additional recA1 mutation, on the other hand, behaves quite differently, its germinated spores being actually more UV sensitive than spores at low UV fluences. There is a fraction of the population, both in early germinated spores of UVSSP-42-1 and in germinated spores of G1, which is much more resistant than the rest of the population.

Fate of photoproducts. Spores of strains UVSSP-42-1 and UVSSP-1-1 were incubated for 10 min at 37°C in germination medium and exposed to UV, and the contents of acid-soluble and acid-insoluble photoproducts were measured during further incubation. The results are shown in Fig. 3. In both strains the cyclobutane-type dimers are removed from DNA,

![Fig. 1. Formation of thymine photoproducts in germinating spores of B. subtilis UVSSP-42-1 as a function of germination time. Spores were placed in germination medium at 37°C and allowed to germinate for various times. Small portions of each sample were used to determine UV survivals as shown in Fig. 2A, and the remaining samples, irradiated with a fixed fluence of 720 J/m², were used to analyze for thymine photoproducts. The two isolated points (×, ○) show analyses for the similarly irradiated vegetative cells of UVSSP-42-1.](http://jb.asm.org/)
whereas all the TDHT remains in trichloroacetic acid-insoluble form. The cyclobutane-type dimers removed are stoichiometrically recovered in the trichloroacetic acid-soluble fraction (Fig. 3A1 and 3B1), indicating that an excision-type repair is responsible for their removal. This is despite the fact that these strains carry mutation hcr₁ or mutation hcr₄2, either of which makes them unable to excise cyclobutane-type dimers in the vegetative phase. We refer to this transient excision repair as germinative excision repair. The transient excision of cyclobutane-type dimers occurs also in the presence of the spore repair process, as

![Graph](http://jb.asm.org/)

**Fig. 2.** Survival of spores, germinated spores, and vegetative cells of B. subtilis strains (A) UVSSP-42-1, (B) UVSSP-1-1, and (C) G1 as a function of UV exposure. Spores were kept in germination medium at 37°C for 0 min (◇), 5 min (+), 10 min (▲), 20 min (●), 30 min (△), or 60 min (○) before the start of irradiation. Vegetative cells (×) and germinated spores were irradiated in 0.1 M potassium phosphate buffer, pH 7.2, at 4°C.

![Graph](http://jb.asm.org/)

**Fig. 3.** Thymine photoproduct removal and general DNA degradation in 10-min germinated spores of B. subtilis strains UVSSP-42-1 (A), UVSSP-1-1 (B), UVSS-42 (C), and G1 (D), irradiated with 720 J/m². Figures 3A1 to DI show the percentage of total thymine radioactivity present as TDHT (right-hand scale, △) and as cyclobutane-type dimers C<>T + T<>T (left-hand scale, ○) after further incubation for the indicated times in 0.1 M potassium phosphate buffer containing 1 mg of glucose per ml. (——) refers to trichloroacetic acid-insoluble fractions, and (------) refers to trichloroacetic acid-soluble fraction. Figures 3A2 to D2 show the percentage of total thymine radioactivity (left-hand scale) present in all forms which is trichloroacetic acid soluble (- - - -) after incubation for the indicated times and the ratio of trichloroacetic acid-soluble thymine to trichloroacetic acid-soluble C<>T + T<>T radioactivity (right-hand scale, - × -).
seen in Fig. 3C1 for strain UVSS-42 (hcr42). This latter process removes TDHT without making it acid soluble (16), apparently converting it back to two thymines (Wang and Rupert, in press). We have observed the patterns of DNA photoprotein elimination in all other strains used in this paper, as well as in the repair-proficient strain UVR, and note that they are consistent with a simple superposition of whatever repair processes (spore repair, vegetative excision repair, or germinative excision repair) are not blocked by mutation.

In Fig. 3A2–D2, we have shown the fraction of total thymine radioactivity rendered trichlo-roacetic acid soluble (left-hand scale) and the ratios of total thymine to cyclobutane-type dimers (C<>T and T<>T) in the trichloroacetic acid-soluble fraction (right-hand scale). In strain UVSSP-1-1, where the incision step of the vegetative excision repair is blocked, the ratio of acid-soluble thymine radioactivity to total acid-soluble dimer activity remains constant with time at about 0.33. From this information, knowing also the relative yield of C<>T and T<>T dimers (1 C<>T:1.65 T<>T) and the guanine-cytosine content (43%) in B. subtilis DNA, we can estimate that only about four nucleotides in all become acid soluble for each thymine-containing dimer excised from this strain, i.e., two in addition to those in the dimer. This means that the germinative excision is remarkably specific for dimer-containing regions. In strains UVSSP-42-1 and G1, where incision but no excision occurs in vegetative cells, a constant ratio of thymine to dimers occurs over about the first hour, corresponding to about seven nucleotides removed per dimer.

Host cell reactivation of irradiated M2 phages. Since the mutations hcr42 and hcr1 block host cell reactivation as well as excision repair in the vegetative cells of B. subtilis, the question arises whether germinated spores of these mutants become capable of host-cell-reactivating UV-irradiated phages. We found that ungerminated spores are not infectable by bacteriophage M2, nor are spores germinated in germination medium or in the complex mediaplus-chloramphenicol used by Munakata (12). However, infectability of spores germinated and grown in BHI or TYB begins after about 40 to 60 min of incubation at 37°C. Although this is later than the time of peak UV resistance, the cells have by no means reverted to vegetative-cell sensitivity (12) and still can excise dimers. We therefore tested the host cell reactivation capacity of 60-min germinated spores. Irradiated phage M2 survives considerably better in spores germinated and grown for 60 min than in vegetative cells of strains carrying either the hcr1 or hcr42 mutation (Fig. 4A and B). These phage survival curves have two components: 5 to 10% of the population behave like phage plated on HCR+ vegetative cells (compare dashed-line extrapolates of Fig. 4A and B with Fig. 4C), and the remainder behave more like phage plated on vegetative cells of HCR− strains (being only 33% more resistant). The fraction of the infected germinated spores which is HCR+ gradually diminishes with time of growth, and finally the sensitivity of the whole population approaches that of the vegetative cells (Fig. 4A and B).

For comparison we have shown also phage survivals on germinated spores of a strain which is hcr+ in the vegetative phase (Fig. 4C). It is evident that irradiated phages survive better in the germinated spores of this strain than in its vegetative cells, arguing that the germinative excision repair is active, along with the constitutive host cell excision system, in these germinated spores.

Relation of recA1 mutation to germinative excision repair. We noted above that germinated spores of strain G1 (hcr42 ssp, recA1) are greatly reduced in their resistance to UV compared with the parental strain UVSSP-42-1 (Fig. 2A and C). This might raise the question whether the germinative excision repair involves the recA1 gene product. However, as seen by comparing Fig. 3A with 3D, photoproduc tion removal is unaffected by the recA1 mutation, and is not accompanied by enhanced DNA degradation. Moreover, the capacity for host cell reactivation in germinated spores is also unaffected (compare Fig. 4A with 4D). In both respects, strain G1 behaves indistinguishably from its parental strain UVSSP-42-1, indicating no involvement of recA1 in germinative excision repair. It seems likely that the extra sensitivity coming with the recA1 mutation may be due to its interference with a supportive recombination repair (19).

DISCUSSION

Changes of a cell's resistance to UV at a particular growth phase may be due to changes in either the quality and/or quantity of UV-induced damages to the cell or to changes in the effectiveness of cellular repair systems which function in different growth phases. In the development of wild B. megaterium from spores to vegetative cells (a germination process which can be highly synchronized), Stafford and Donnellan (23) have shown that the transitory UV resistance is correlated with a period of low production of total thymine photoproducts. In B. subtilis, on the other hand, we find that
Fig. 4. Survival of B. subtilis phage M2 as a function of UV fluence when plated on germinated spores and vegetative cells of strains UVSSP-42-1 (A), UVSSP-1-1 (B), SSP-1 (C), and G1 (D). Phages were exposed to UV in 10 mM tris(hydroxymethyl)aminomethane (pH 7.5), plus 0.09 M NaCl, at about 5 × 10^7 phages/ml. They were adsorbed to vegetative cells (x) or to spores germinated and grown in BHI for 60 min (○) or 120 min (△), before being plated as described under Materials and Methods. The first cell division occurs about 150 min after germination and growth in BHI at 37°C.

High UV resistance in germinated spores of an HCR^- strain occurs without a drastic reduction in the amount of photoproduct formed and can be attributed instead to the presence of an additional transitory germinative excision repair system. This excision repair excises only the cyclobutane-type dimers, in contrast to the constitutive excision repair active in the vegetative cells (controlled by genes hcr1, hcr42, etc.), which will excise both TDHT and cyclobutane-type dimers some time after germination (15).

At present we are not certain whether a very low production of thymine photoproducts in a small part of the population is responsible for the fraction of germinated B. subtilis spores showing highest UV resistance (see the resistant tail in Fig. 2) because germination of the population is nonsynchronous. Likewise, we do not know whether germinative excision repair is present in spores of B. megaterium or B. cereus, because we currently lack comparable HCR^- mutants in these species. It may be noted that, in the excision-proficient wild-type spores of B. megaterium, TDHT was apparently removed only by spore repair (2), since there was no indication of the acid solubilization that would be expected if appreciable excision occurred. This contrasts with the dual removal
mechanisms for TDHT which are evident in *B. subtilis* and *B. cereus* (16). It is possible that the vegetative excision repair system of *B. megaterium*, like the germinative excision repair in *B. subtilis*, excises only cyclobutane-type dimers.

Our current understanding of excision repair of UV-induced cyclobutane-type pyrimidine dimers pictures a four-stage process (for reviews, see 5, 8, 18, 20). The early steps of this process involve endonucleolytic incision in the vicinity of the dimer, followed by exonucleolytic removal of the dimer and several adjacent nucleotides. Two different *B. subtilis* HCR 

mutants, which are defective for removal of cyclobutane-type dimers from vegetative cell DNA and also for the slow excision of TDHT from spore DNA (16), are able to excise cyclobutane-type dimers formed by irradiation in the germination phase. Since one of these mutations (hcr1) blocks the initial incision step, whereas the other (hcr2) blocks some later step of the removal by the vegetative excision mechanism, the germinative excision repair must utilize enzymes different from the vegetative mechanism for at least these two steps.

Germinative excision repair is very precise in excising dimers (Fig. 3A2–D2), compared with the constitutive system that normally functions in the vegetative phase (21). The two additional nucleotides excised along with each dimer (making a total of four nucleotides removed) from the strain UVSSP-1-1 is much smaller than that for the vegetative excision repair in removing either cyclobutane-type dimers (21) or TDHT (16). It approximates the size estimated for the distorted region of DNA caused by a dimer (6), and agrees well with the size of fragments removed during in vitro excision of cyclobutane-type dimers (4).

It is understandable why spores might need to be equipped with extra repair systems to protect them from UV damage to ensure their outgrowth. In nature they are likely to be exposed to solar radiation from time to time while metabolically inert and unable to carry out repair. Under these circumstances they could accumulate a heavy burden of photoproduct (TDHT) over the period of time before germination. In addition, upon germination, the germinated spore might sometimes find itself in an environment where nutrients were insufficient for rapid development to the fully functioning vegetative state. If exposed to more solar radiation under these circumstances, the germinated spore would accumulate further DNA photoproducts, in this case predominantly cyclobutane-type dimers. The spore repair and the germinative excision repair processes have exactly the characteristics needed to overcome this pair of problems.

The fact that the germinative excision repair, like the spore repair process (16), occurs only transiently and is not observed in vegetative cells shows that repair enzymes are not all synthesized constitutively. It also illustrates how an organism capable of a kind of cell differentiation may be provided with different mechanisms for repairing UV damages incurred at various stages of development and how these mechanisms may have different photoproduct specificities. It suggests that such a regulation of DNA repair enzymes might also occur in the differentiating cells of higher organisms.

ACKNOWLEDGMENT

This work was supported by U. S. Public Health Service research grant GM 16547 from the National Institute of General Medical Sciences.

LITERATURE CITED


13. Munakata, N., P. C. Fitz-James, and I. E. Young. 1975. Ultraviolet sensitivity and photoproducts in spore-like bodies of an excision-repair deficient and


