EFFECT OF MITOMYCIN C ON FIVE EXCISION-REPAIR MUTANTS OF *BACILLUS SUBTILIS*

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Bacterial mutants deficient in any of the excision-repair enzymes responsible for the dark repair process are generally characterized by extreme sensitivity to ultraviolet irradiation and mitomycin C, both of which cause specific lesions in DNA. Sensitivity to both agents may result from a single mutational event (Okubo and Romig, 1966).

Iyer and Szybalski (1963) showed that mitomycin causes crosslinking of DNA both *in vivo* and *in vitro*. The extent of crosslinking can be measured by heat denaturing mitomycin-treated DNA; all crosslinked molecules undergo reversible denaturation.

Transformation studies carried out with DNA isolated from a mitomycin treated wild-type strain and five excision-repair (ER^-) mutants of *B. subtilis* showed that mitomycin causes a considerable degree of crosslinking in all the strains examined. Transforming DNA extracted from the wild-type strain and from one of the mutants which was host cell reactivating (hcr^+) showed a marked increase in heat denaturability after the mitomycin-treated cells had undergone a 30-minute recovery period. DNA isolated from four other ER^- strains remained crosslinked over a recovery period of 2 hours.

Materials and Methods. Mitomycin C treatment -- Cells were grown in brain heart infusion broth (Difco) to a viable count of 4×10^8 cells/ml and were treated for 5-15 minutes with 1 μg/ml of mitomycin C obtained from the Kyowa Hakka Kogyo Co., Ltd.

Isolation of DNA -- Mitomycin treated cells were centrifuged in the cold, washed twice with saline-phosphate buffer (pH 7), resuspended in 0.15 M NaCl-0.1 M EDTA (pH 8.2), and lysed with 100 μg/ml of lysozyme. Protein was removed by cold phenol treatment (Kirby, 1958). DNA was alcohol precipitated following a 20-minute incubation (37°) with 50 μg/ml of RNAse (Worthington Biochem. Corp.).

Transformation -- The preparation of competent cells and transforming procedures have been described previously, (Mahler et al., 1963). Denatured transforming DNA was prepared by heating DNA at 20 μg/ml in 0.015 M NaCl-0.0015 M trisodium citrate to 100° for 10 minutes, followed by rapid chilling in ice-water.

Results. Derivation and Characteristics of Mutant Strains -- Four ER^- (excision-repair) strains were isolated on the basis of sensitivity to ultraviolet (UV) irradiation as described previously (Mahler 1965). ER_4, isolated on the basis of mitomycin sensitivity, was a gift from Dr. Julius Marmur. ER_2, ER_4 and ER_9 were derived from B. subtilis 168^{wt}, ER_1 from 168 $tryptophan_2^-$ and ER_{20} from 168 $thymidine^-$, $tryp_2^-$. The strains in order of increasing sensitivity to 1400 ergs/mm^2 of UV light are ER_9, ER_2, ER_{20}, ER_1, ER_4. All five mutants failed to grow on brain heart infusion agar plates containing 0.055 μg/ml of mitomycin, a concentration which did not inhibit the parental strains.

Figure 1 shows the survival of irradiated phage SP82 plated on B. subtilis 168^{wt} and on four of the ER^- mutants. ER_2 no longer adsorbed SP82. Of the four UV^-, mitomycin-sensitive mutants, only ER_9 was capable of host cell reactivating (hcr^+) irradiated SP82 phage.

DNA from the slow-growing strain ER_4 was effective in transforming SB-1 receptor cells, but competent ER_4 cells were found to yield few if any transformants with bacterial DNA and no plaques with DNA isolated from phage SP82. Since recombination is required for expression of transformants and of infectious SP82 DNA (Green, 1964), it seemed possible that ER_4 might be a recombination-deficient strain. However, studies with SB-1 and ER_4 both made competent at equivalent cell concentration and exposed to tritiated transforming DNA

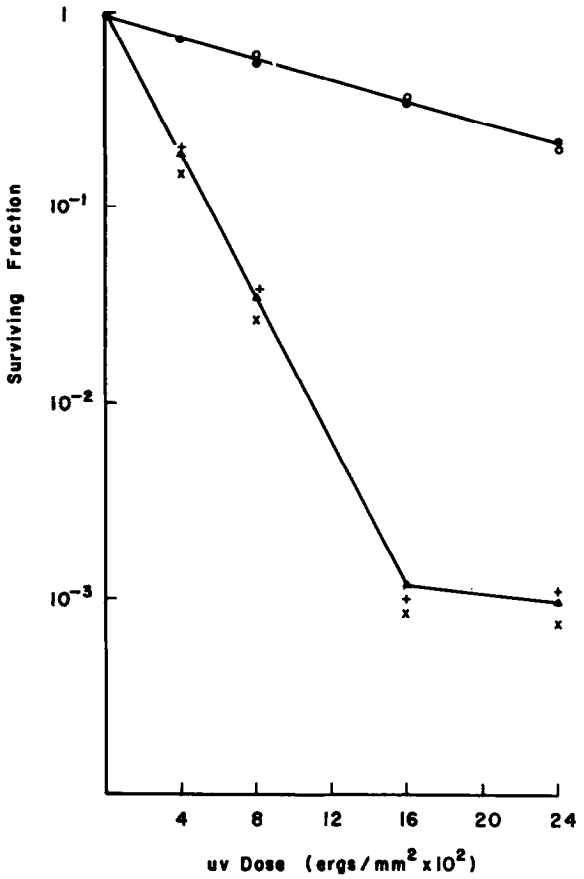

Fig. 1. Host cell reactivation of UV irradiated SP82 phage. SP82 at a concentration of 2.4 x 10^8 particles/ml was irradiated in 0.1 M Tris buffer and plated on tryptone agar containing 1.3 µg/ml of $MnCl_2$. (•) on host 168^{wt}; (Δ) ER_1; (x) ER_4; (o) ER_9; (+) ER_{20}.

showed that ER_4 is probably blocked at the level of DNA uptake, since it incorporated only 10% of the radioactivity taken up by SB-1.

Mitomycin treatment — The survival of the five ER^- strains and of two wild-type strains treated with mitomycin C is shown in Table I. The sensitivity to UV and mitomycin is parallel in all five mutants. Since the survival of cells exposed to UV or mitomycin may be influenced by factors other than DNA excision and repair, it is not surprising that considerable differences in sensitivity to the two agents may be discovered (Terawaki and Greenberg, 1966).

Table I

Survival of Bacillus subtilis strains after Mitomycin C treatment

Cells grown in broth were treated with mitomycin for 15 minutes at 37° with shaking. Treated cells were diluted and plated on brain heart infusion agar plates.

Strain	Fraction of survivors
	Mitomycin C (1μg/ml)
168^{wt}	4×10^{-3}
168 thy^-, $tryp_2^-$	2.3×10^{-3}
ER_9	1.7×10^{-4}
ER_2	9×10^{-5}
ER_{20}	6.6×10^{-5}
ER_1	1.6×10^{-5}
ER_4	5.4×10^{-6}

Table II

Transformation with DNA isolated from mitomycin-treated and untreated B. subtilis cells

All transformations were carried out with 2 μg/ml of DNA. The numbers of transformants were averaged from three separate experiments.

Recipient	168^{wt} DNA	Histidine Transformants	Residual Transformation
SB-1	Native	5.2×10^5	
	Denatured	1.0×10^4	2%
	Mitomycin treated Native	4.5×10^5	
	Mitomycin treated Denatured	2.6×10^5	58%

For transformation experiments, DNA was extracted from cells treated with 1 μg/ml of mitomycin for 5-15 minutes. DNA treated *in vitro* with reduced mitomycin or isolated from mitomycin-treated cells shows only a small decrease in transforming efficiency. The yield of histidine transformants with native and heat-denatured DNA isolated from untreated cells of 168^{wt} is compared to transformants obtained with DNA isolated from mitomycin-treated cells in Table II. The high transforming efficiency following heat denaturation of DNA obtained from mitomycin-treated cells reflects the effective crosslinking of a large portion of the DNA.

Boyce and Howard-Flanders (1964) reported that a mitomycin-treated wild type strain of *Escherichia coli* K12 was capable of excising fragments of DNA while three UV^- mutants were not. If crosslinks can undergo intracellular excision in certain bacterial strains, the susceptibility to heat denaturation of DNA isolated from mitomycin-treated *B. subtilis* cells should increase in the wild-type strain but remain unchanged in ER^- mutants.

For recovery experiments, treated cells were washed free of mitomycin, resuspended in broth and permitted to undergo recovery periods of 30 minutes to 2 hours. *B. subtilis* 168 is known to carry a defective prophage, PBSX, which is inducible by mitomycin (Seaman *et al.*, 1964) (Ionesco *et al.*, 1964). However, mitomycin added to cultures in the late logarithmic or early stationary phase of growth generally does not result in cell lysis (Seaman, personal communication).

Figure 2 shows the residual transforming activity after heat denaturation of DNA isolated from wild-type and mutant *B. subtilis* strains. All strains were treated with mitomycin for a period of 15 minutes, then permitted to recover for a total period of 2 hours. The degree of mitomycin-induced crosslinking (resulting in high residual transformation) is virtually complete after 5 minutes of treatment. The disappearance of crosslinks, resulting in enhanced susceptibility to heat denaturation, is evident after 30 minutes of recovery in DNA from the wild-type strain and ER_9 which is hcr^+. After a recovery period of 2 hours, DNA isolated from the four hcr^- mutants showed only a minor increase in heat denaturability.

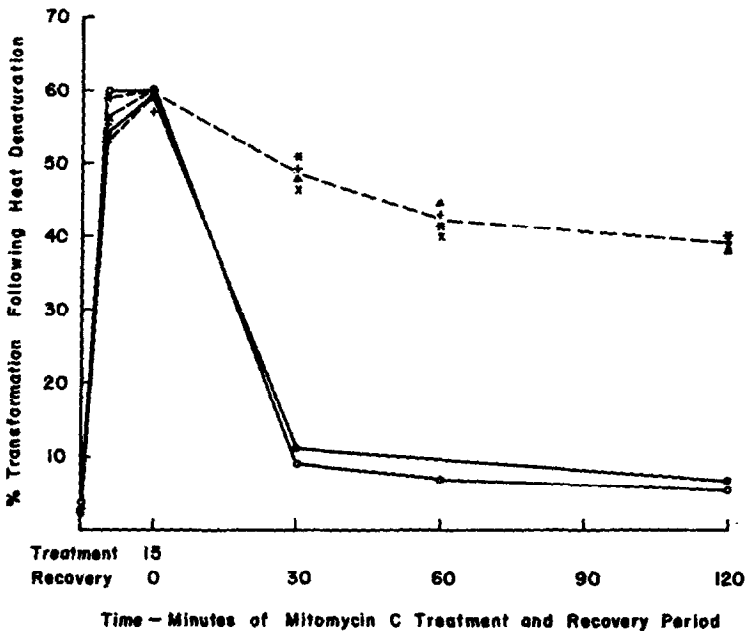

Fig. 2. % residual transforming activity of heat-denatured DNA isolated from six strains of B. subtilis after mitomycin treatment and after recovery periods. Competent SB-1 cells were transformed to histidine$^+$ with 2 μg/ml of native and heat-denatured DNA from: (•) 168^{wt}; (o) ER_9; (▲) ER_1; (*) ER_2; (x) ER_4; (+) ER_{20}.

Discussion -- Bacterial hcr$^+$ strains capable of excising pyrimidine dimers might also be able to excise mitomycin-induced crosslinks. Of the five UV$^-$, mitomycin sensitive mutants of B. subtilis examined, only ER_9, which was found to be hcr$^+$, showed a loss of interstrand DNA crosslinks during a recovery period. The residual transforming activity, following heat denaturation, of ER_9, DNA after a 30 minute recovery period was identical to the activity of DNA isolated from a wild-type control strain. The disappearance of crosslinks in DNA from hcr$^+$ strains of mitomycin-treated E. coli B has been reported by Terawaki and Greenberg (1966). Although the host cell reactivation of ER_2 could not be tested, it is presumably hcr$^-$, as are ER_1, ER_4 and ER_{20}, since no excision of mitomycin crosslinks occurred in DNA isolated from these strains.

As suggested previously (Boyce and Howard-Flanders, 1964), the recovery of DNA from mitomycin damage may occur in a manner similar to the recovery from UV

radiation: an endonucleolytic cut at the site of configurational distortion with exonucleolytic enlargement followed by repair with nucleotides complementary to the unexcised strand.

We have obtained evidence (Dolbeare et al., to be published) that a purified enzymatic fraction from Micrococcus lysodeikticus with no nucleolytic activity toward native transforming DNA can degrade both UV irradiated and mitomycin-treated DNA. This suggests that similar enzymatic events may initiate excision of both UV and mitomycin-induced DNA damage.

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