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Repair of Ultraviolet Light-Induced Damage in *Micrococcus radiophilus*, an Extremely Resistant Microorganism

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Repair of ultraviolet radiation damage was examined in an extremely radioresistant organism, *Micrococcus radiophilus*. Measurement of the number of thymine-containing dimers formed as a function of ultraviolet dose suggests that the ability of this organism to withstand high doses of ultraviolet radiation (20,000 ergs/mm^2) is not related to protective screening by pigments. *M. radiophilus* carries out a rapid excision of thymine dimers at doses of ultraviolet light up to 10,000 ergs/mm^2. Synthesis of deoxyribonucleic acid is reduced after irradiation, but after removal of photodamage the rate approaches that in unirradiated cells. A comparison is drawn with *Micrococcus luteus* and *M. radiodurans*. We conclude that the extremely high resistance to ultraviolet irradiation in *M. radiophilus* is at least partly due to the presence of an efficient excision repair system.

Sensitivity to ultraviolet (UV) light appears to result primarily from the inability of cells to cope with damage to deoxyribonucleic acid (DNA) (28, 31). Ability to survive moderate doses of UV radiation can be enhanced by irradiation with visible light (26), by removal of lesions from DNA in the dark (11, 25, 30), or by recombination events between sister chromatids (23, 24). One or more of these mechanisms may operate in a single organism (5, 6). However, it is still not certain whether a more efficient removal process or a greater coordination between different repair systems confers on a particular organism a high level of resistance to UV light.

Several species of *Micrococcus* have been shown to be highly resistant to UV radiation (7, 29). The most studied of these is *Micrococcus radiodurans*, which is thought to owe its resistance largely to an efficient, rapid removal of pyrimidine dimers from DNA after UV radiation (1). This organism also shows a high level of resistance to X-rays (8). Recently, another species, *Micrococcus radiophilus*, which is resistant to UV radiation (13) and to X-rays (12) has been isolated. The quantitation of survival after exposure to UV light indicates that this species is somewhat more resistant than *M. radiodurans* (13).

The present paper reports investigations on the nature of UV-induced damage in *M. radiophilus* and its repair, in comparison with *M. radiodurans* and the more sensitive species, *M. luteus*.

MATERIALS AND METHODS

Cultures. *M. radiophilus* (NCTC 10785) was obtained from the National Collection of Type Cultures (NCTC), London, and *M. luteus* from the Department of Microbiology, University of Queensland. Cultures were grown in TGYM medium containing 0.5% tryptone, 0.2% glucose, 0.3% yeast extract, and 50 mg of DL-methionine.

Survival curves. Cells were grown either at 30 C (*M. radiophilus*) or at 37 C (*M. luteus*) in TGYM medium. Exponentially growing cells were centrifuged and resuspended in 0.1 M phosphate buffer (pH 7.2) at a concentration of approximately 10^8 cells/ml. Samples, usually 1 ml, were placed in a bacterial plate on ice and irradiated at 254 nm with constant stirring. Intensity of the incident UV light was measured using an ultraviolet intensity meter (Ultra-Violet Products, Inc., San Gabriel, Calif.). Colonies were counted after a 3-day incubation at 30 C.

Estimation of single- and double-strand breaks. Double-strand breaks in DNA were examined by sedimentation in linear gradients of 5 to 20% (wt/vol) sucrose in 0.9 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.5), and 0.01 M disodium ethylenediaminetetraacetate. Single-strand breaks were determined under the same conditions, except that 0.1 M NaOH was substituted for Tris buffer. Cells were lysed by incubating with lysozyme (200 μg/ml) for 15 min in 0.01 M Tris (pH 7.5) at 37 C, followed by the addition of 1% sodium lauryl sarcosine. A 0.1-ml sample of lysate from cells labeled with [^{3}H]thymidine (10 μCi/ml, 19.2 Ci/mmol) for 5 h was layered on the appropriate sucrose gradient, and the latter was centrifuged at 35,000 rpm at 4 C in the SW56 rotor in a Beckman L2-65B ultracentrifuge.

Assay of thymine dimers. The dimer content of irradiated DNA was measured using two-dimensional paper chromatography as described by Carrier and Setlow (4).

Effect of UV radiation on synthesis of DNA. Incorporation of [^{3}H]thymidine into acid-insoluble material was taken as a measure of DNA synthesis. Overnight cultures were centrifuged and resuspended in phosphate-buffered saline (21) at a density of about 10^8 cells/ml prior to irradiation. Cells were again centrifuged, resuspended in TGYM medium, and incubated. Samples were removed every hour and labeled with [^{3}H]thymidine for 30 min, and the reaction was terminated by adding an equal volume of ice-cold 10% trichloroacetic acid. The samples were collected on glass filters (GF/C Whatman) and counted by liquid scintillation spectrometry in a toluene phosphor, containing 4 g of diphenyloxazole and 50 mg of dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)benzene] per liter of toluene.

Preparation of cell extracts. *M. radiophilus* cells were grown overnight, centrifuged, and resuspended in 0.01 M potassium phosphate buffer, pH 7.5. The cells were divided into two fractions, one of which was stored at −20 C while the other was irradiated with UV light. The irradiated sample was centrifuged, resuspended in TGYM medium, and incubated for 30 min at 37 C prior to storage at −20 C. *M. luteus* cells were prepared in the same manner. Cell suspensions containing 10^9 cells/ml were sonicated for 2.5 min (5 × 30 s) using an MSE ultrasonic power unit with the temperature maintained below 10 C. This was followed by centrifugation at 15,000 × *g* for 20 min, with the supernatant solutions being retained for enzyme assays.

Enzyme assays. For assay of endonuclease activity, bacteriophage T3 DNA, labeled with [^{3}H]thymidine (1 μCi/ml), was prepared according to the method of Thomas and Abelson (34). Reaction mixtures contained 0.8 μg of ^{3}H-labeled DNA, irradiated in solution with 6,000 ergs/mm^2, 0.05 M Tris buffer (pH 8), 0.1 M $MgCl_2$, and cell extract in a total volume of 0.1 ml. At this dose, about 1.5% of thymine residues were dimerized. Incubation was carried out at 37 C for various times, and the reactions were terminated by the addition of 0.1 volume of 1% sodium lauryl sarcosinate. Samples were then applied to sucrose gradients as described above. Fractions were collected on filter paper (Whatman no. 1), dried, and counted by liquid scintillation spectrometry in a toluene phosphor. Reaction conditions were the same where activity against normal DNA was determined. Relative molecular weight using intact T3 DNA as substrate was calculated according to Burgi and Hershey (2). DNA polymerase activity was determined by the method of Englund (9). Exonuclease activity was determined by measuring the production of acid-soluble nucleotide under the incubation conditions used for assay of endonuclease.

RESULTS

Cell survival. A comparison of the survival curves for *M. radiophilus* and *M. luteus* following UV radiation appears in Fig. 1. The results

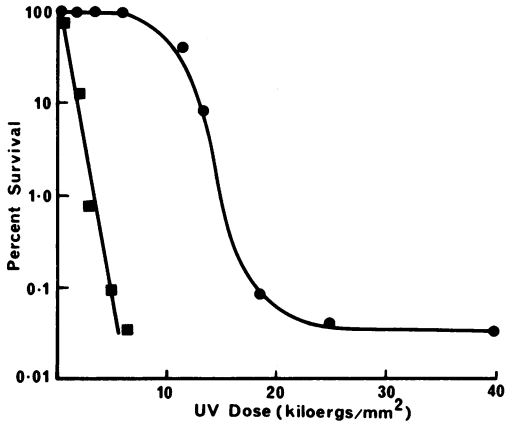

FIG. 1. *Bacterial survival in response to UV irradiation. Exponentially growing cells were suspended in phosphate buffer and irradiated in a thin film (0.3 mm) of liquid. Symbols: ●, M. radiophilus; ■, M. luteus.*

indicate that the shoulder in the survival curve of *M. radiophilus* is about an order of magnitude greater than that for *M. luteus*, suggesting that it possesses a more efficient means of repair of photo-damage. *M. radiodurans* (27) is somewhat less resistant to UV than is *M. radiophilus*. *M. radiophilus* exhibited a very large shoulder on its survival curve. In addition the curve had a drastically different slope at UV exposures greater than 20,000 ergs/mm^2, where survival was reduced to 0.01% (Fig. 1) in agreement with published data (13). This change in shape in the survival curve was not apparent in the case of the other organisms.

Dimer formation. The ability to withstand such high doses of UV radiation indicates that the organism is capable of absorbing the majority of the UV light utilizing chromophores at the level of the cell wall, membrane, or cytoplasm, that it possesses an efficient system which is capable of removing lesions in its DNA rapidly, or that it uses other mechanisms to circumvent damage. Dimer formation (Fig. 2) was found to be linear up to about 16,000 ergs/mm^2, leveling off with about 4% thymine as dimers at 30,000 ergs/mm^2. Comparison with *M. radiodurans* (29) indicated that a high level of dimer formation also occurred in *M. radiophilus*.

Dimer removal. At UV doses up to 8,000 ergs/mm^2, where 1.8% of thymine residues are present as dimers, *M. radiophilus* showed 100% survival as measured by colony formation on agar (Fig. 1). Survival presumably depends to a significant extent on removal of these and other photo products, since it is very unlikely that it could tolerate such a high number of dimers in

its DNA for very long. At 5,000 ergs/mm², on the shoulder of the survival curve, 50% of dimers were removed in 30 min, and essentially all dimers were removed in 2 h (Fig. 3). When the dose was doubled to where survival was about 50%, the rate of removal of dimers was reduced to approximately half that at 5,000 ergs/mm², and residual dimers were measurable after 4 h. When the dose was raised to a level which resulted in 0.1% survival, the rate of dimer removal slowed considerably.

Single-strand breaks. Single-strand breaks made after UV irradiation were demonstrated by alkaline sucrose gradient analysis (15). Cells were labeled with [³H]thymidine (10 μCi/ml) for 5 h prior to irradiation, equivalent to 2 to 3 rounds of replication. After a 30-min incubation, after UV irradiation, single-strand breaks were detected in *M. radiophilus* DNA (Fig. 4). However, after 3.5 h of incubation the size of DNA from irradiated cells had almost reached that of unirradiated cells (Fig. 4). No double-strand breaks were detected under these conditions.

Effect on replication. Since UV radiation has been shown to inhibit or slow down replication in several organisms studied (16, 29, 32, 33), the ability of this organism to maintain normal levels of replication after exposure to UV radiation was determined. At a UV dose of 600 ergs/mm² little or no effect was observed on the rate of incorporation of [³H]thymidine into DNA in *M. radiophilus* (Fig. 5A). DNA synthesis in *M. luteus* was also unaffected at this UV dose (Fig. 5B). When *M. radiophilus* was exposed to a dose of 5,000 ergs/mm² a delay of approximately 4 h was observed in the synthesis of DNA after which synthesis appeared to proceed at the normal rate (Fig. 6). Further increases of the UV dose led to greater lags in DNA synthesis. Synthesis of DNA in *M. luteus* was completely abolished at UV doses over 3,500 ergs/mm².

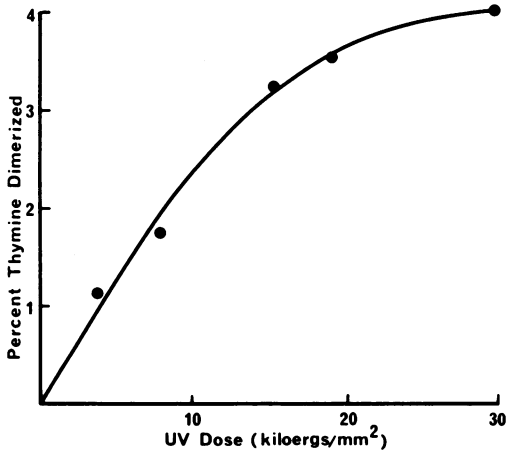

FIG. 2. *Formation of thymine-containing dimers in DNA of M. radiophilus after UV irradiation. Acid-insoluble dimers were measured using two-dimensional paper chromatography (4).*

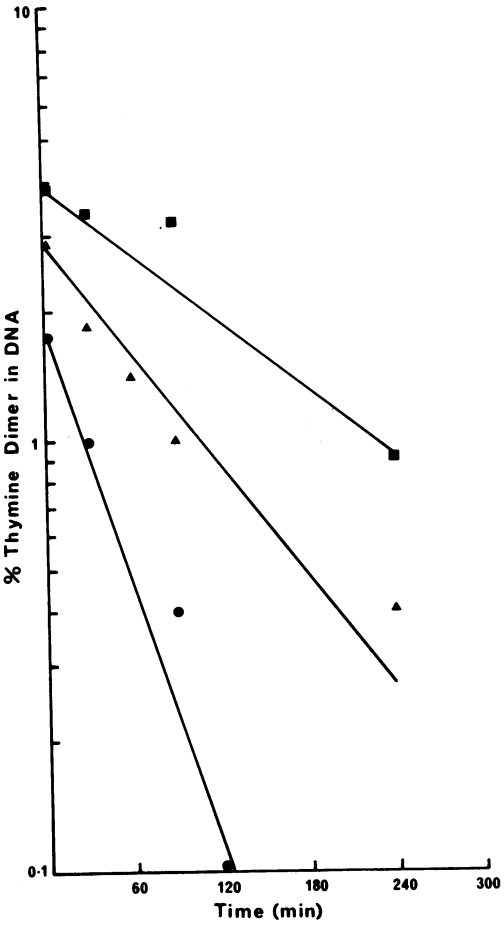

FIG. 3. *Removal of thymine-containing dimers from DNA of M. radiophilus. Cells were irradiated in phosphate buffer and subsequently incubated in TGYM medium at 30 C for the times indicated. Samples were withdrawn, and acid-insoluble dimers, remaining with time, were determined by chromatography. Symbols (UV dose): ●, 5,000 ergs/mm²; ▲, 10,000 ergs/mm²; ■, 18,000 ergs/mm².*

Enzyme activities. Endonuclease activities from extracts of previously irradiated (5,000 ergs/mm²) and unirradiated *M. radiophilus* were compared to those obtained in *M. luteus* using bacteriophage T3 DNA as substrate. Incubation with extracts of *M. radiophilus* resulted in a decrease in molecular weight with time. When UV irradiated (6,000 ergs/mm²) T3 DNA was used as substrate no change in the

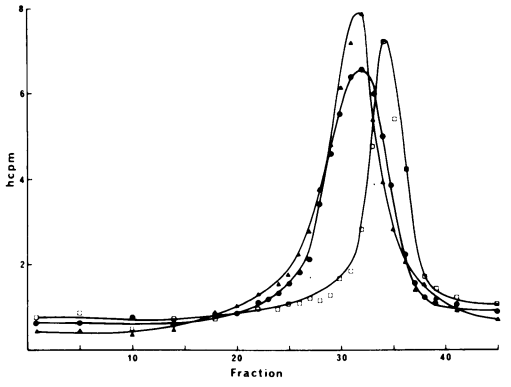

FIG. 4. *Sedimentation profile of M. radiophilus DNA after exposure of cells to UV (3,600 ergs/mm²). Cells were labeled with [³H]thymidine, irradiated, and incubated for various times in TGYM medium at 30 C prior to centrifugation in 5 to 20% alkaline sucrose gradients for 90 min at 35,000 rpm in a Spinco SW56 rotor at 4 C. Five-drop fractions were collected from the bottom of the tube, precipitated with 0.1 ml of 10% trichloroacetic acid, washed in ethanol, and radioactivity was counted. The molecular weight of the unirradiated DNA was 1.6 × 10⁸. Symbols: ▲, unirradiated cells; □, cells irradiated and incubated in medium for 30 min; ●, cells irradiated and incubated for 3.5 h. Sedimentation is from right to left.*

rate of decrease of molecular weight was achieved. Extracts of *M. radiophilus* which had been irradiated prior to lysis gave similar results to the unirradiated extracts. Incubation of extracts of *M. luteus* with DNA under the same conditions also caused a decrease in the molecular weight of the DNA, but the rate and extent were somewhat less than those obtained with extracts of *M. radiophilus*. When DNA polymerase and exonuclease activities were tested in the extracts described above no significant differences were observed in material from the three sources.

DISCUSSION

The ability of *M. radiophilus* to withstand high doses of UV irradiation has been investigated. The extent of dimer formation with increasing UV dose is similar to that obtained in *M. radiodurans* (27). This suggests that pigments do not play a role in the observed resistance, which is in agreement with a recent report (14) that qualitative and quantitative variations in the carotenoid pigment of *M. radiophilus* had no effect on resistance to gamma or UV radiation in the organism.

Because of its high resistance to UV it might be expected that *M. radiophilus* is capable of

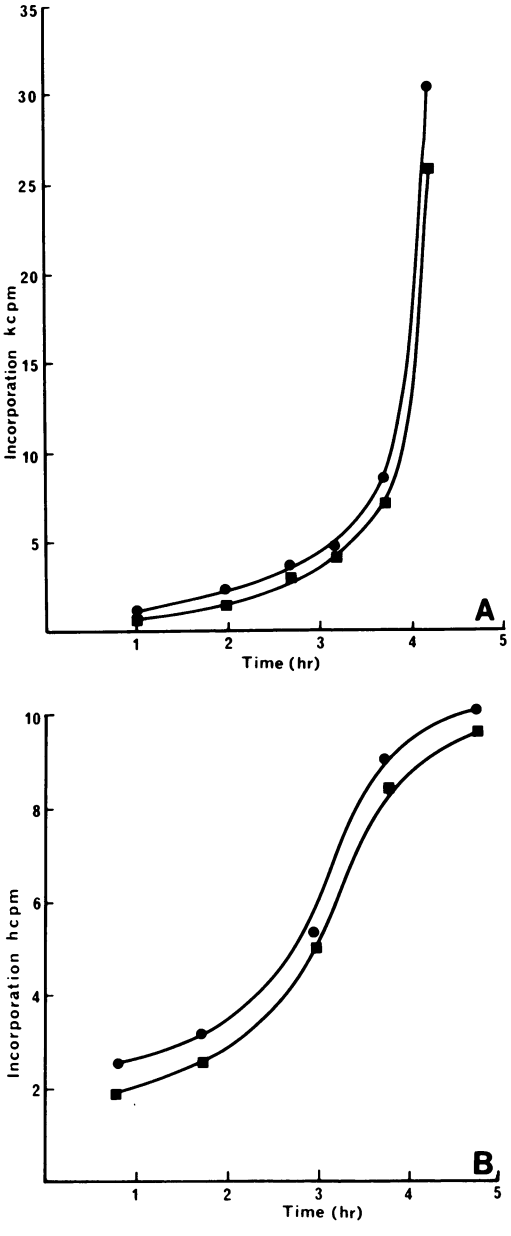

FIG. 5. *Effect of UV irradiation on synthesis of DNA. After irradiation in phosphate-buffered saline cells were incubated in medium at either 30 C (M. radiophilus) or 37 C (M. luteus). Cell samples (1 ml) were pulsed at various times with [³H]thymidine (0.2 μCi/ml) for a period of 30 min, and synthesis was terminated by addition of an equal volume of cold 10% trichloroacetic acid. (A) M. radiophilus. Symbols: ●, unirradiated; ■, irradiated with 600 ergs/mm². (B) M. luteus. Symbols: ●, unirradiated; ■, irradiated with 600 ergs/mm².*

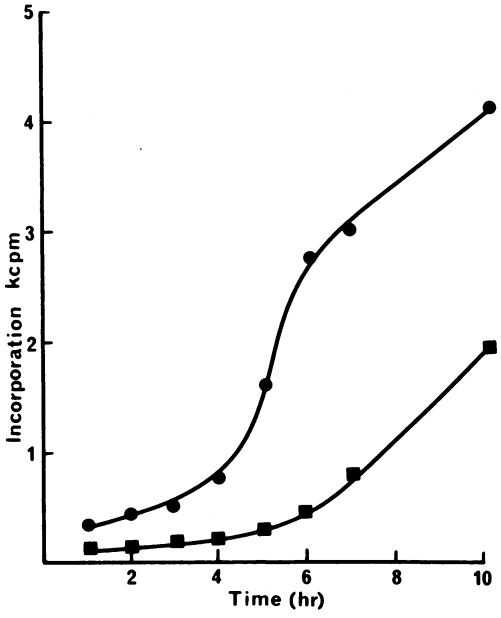

FIG. 6. *Effect of UV irradiation on DNA synthesis in M. radiophilus. Details as in legend to Fig. 5, except that cells were pulse-labeled with [^{3}H]thymidine (0.02 μCi/ml). Symbols: ●, unirradiated cells; ■, cells irradiated with 5,000 ergs/mm^2.*

removing UV damage more rapidly than less resistant organisms. The results in Fig. 3 show that after a UV dose of 5,000 ergs/mm^2, all of the dimers are removed in a period equivalent to about one generation time. At the same UV dose, the rate and extent of removal of dimers was similar for *M. radiodurans* (1). When the UV dose was increased to 10,000 ergs/mm^2 a decrease in the rate of removal of dimers occurred similar to that observed in *M. radiodurans* (1). These results suggest that in *M. radiophilus*, this rapidity of removal of dimers contributes to the extreme resistance observed (Fig. 1). The results in Fig. 4 demonstrate that breakage and reunion of DNA occurs after UV radiation. These breaks may occur exclusively at or near pyrimidine dimers in the process of excision repair. On the other hand, incisions might also occur at other types of excisable photodamage such as that described for polydeoxyadenylic acid (22). The fraction of dimers removed with time (50% in 30 min) in *M. radiophilus* at high doses of UV (5,000 ergs/mm^2) is of the same order as that observed in the same time in more sensitive organisms at low doses (200 ergs/mm^2), where the latter still have considerable colony-forming ability (30).

A delay in DNA synthesis after UV radiation was obtained in *M. radiophilus* at 5,000 ergs/mm^2, which was of the order of that obtained for *M. radiodurans* (27, 29). In both cases all of the dimers had been removed at a time which was half way through this lag period. This delay after dimer removal might be required for the repair of other damage or for the synthesis of proteins necessary for replication. It has been postulated that protein damage is important in the killing and delay in DNA synthesis in *M. radiodurans* (17, 27).

It has been demonstrated that the specific activity of a soluble DNA polymerase in *M. radiodurans* increases in response to X-irradiation (N. E. Gentner, Fed. Proc. 32:1287, 1973). In the present studies using UV radiation, no dramatic changes in either polymerase or nuclease activities were observed in *M. radiophilus* when compared with *M. luteus*. Irradiation of cells followed by incubation prior to extraction failed to induce any further activity. Small changes in activities of individual nucleases or polymerases associated with repair of UV damage would not be detected by the methods used.

We conclude that the ability of *M. radiophilus* to maintain an efficient excision repair system at high doses of UV contributes, at least in part, to the extreme resistance of this organism. The isolation of a mutant from *M. radiodurans* (18, 19), five times more sensitive to UV radiation, with characteristics resembling those of the *exr*$^-$ mutant of *E. coli* (35) indicates a role for postreplication repair involving recombination in the recovery of the wild type from UV damage in addition to that of an effective excision process. The extreme resistance in *M. radiophilus* might be due to an efficient coordination of excision repair and other systems such as post-replication repair; this requires further investigation.

ACKNOWLEDGMENT

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