Effects of $^{60}$Co Gamma-Rays, Ultraviolet Light, and Mitomycin C on Halobacterium salinarium and Thiobacillus intermedius

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Lethal effects of $^{60}$Co γ-rays, UV light, and mitomycin C on two kinds of bacteria, Halobacterium salinarium which grows in highly concentrated salt media and Thiobacillus intermedius which requires reduced sulfur compounds, were studied and compared with those on Escherichia coli B/r. D$_{25}$ values for H. salinarium, T. intermedius and E. coli B/r were 393, 150, and 92 Gy, respectively, by exposure to $^{60}$Co γ-rays. They were 212, 38, and 10 J/m$^2$, respectively, by exposure to UV light and 2.36, 0.25, and 0.53 µg/ml/h, respectively, by exposure to mitomycin C. Against these agents, H. salinarium was much more resistant than T. intermedius and E. coli B/r.

INTRODUCTION

Very highly radioresistant bacterium, Rubrobacter radiotolerans, contains red pigments which are mainly bacterioruberin$^{10}$ with high OH radical-scavenging activity$^{11}$. Bacterioruberin had been also found in Halobacteria$^{14,15}$, represented by Halobacterium salinarium growing in highly concentrated salt media. The first purpose of this study was to determine whether or not this Halobacterium also had highly protective character against ionizing radiation. The second was to compare its resistibility to ultraviolet (UV) light. Presently, photoreactivation to UV light and the lack of dark excision repair are well known in Halobacteria$^{5-9}$, but there is no acceptable explanation, other than the photoreactivation pathway, for its resistibility. We also studied its resistibility to mitomycin C, an inhibitor of DNA synthesis.

It is well known that sulfur compounds protect effectively against radiation$^{10}$. Thiobacteria have an absolute requirement for reduced sulfur compounds$^{11}$. It is interesting to confirm whether Thiobacteria are radioresistant or not. Therefore, one of the mixotrophic chemolithotroph bacteria, Thiobacillus intermedius$^{12}$, was also used in this experiment for comparing its resistibility not only to ionizing radiation but also to UV light and mitomycin C.

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The above two bacteria were compared with Escherichia coli B/r, a radioresistant mutant of Escherichia coli B'3', on the basis of their resistibility against the three types of agents.

MATERIALS AND METHODS

Bacteria

Halobacterium salinarium NRC 34002 was kindly supplied by Dr. S. C. Kushwaha. Complex medium of Sehgal and Gibbons (CM) was used for growth of this bacterium, containing 200 g NaCl, 2 g KCl, 20 g MgSO_{4}.7H_{2}O, 2.3 mg FeCl_{2}.4H_{2}O, 3 g trisodium citrate, 10 g Bacto-yeast extract (Difco), 7.5 g Bacto-casamino acid (Difco) and 8.33 ml glycerol per liter of distilled water. The solutions containing the salts and the organic nutrients were autoclaved separately at 120°C for 10 min, allowed to cool and combined, then adjusted to pH 6.86, and finally autoclaved again at 120°C for 20 min. Cells were grown with shaking at 37°C for 48 h in this fresh medium. Cells at logarithmic phase were collected by centrifugation at 3,500 rpm for 10 min, washed three times, and then resuspended at a concentration of 10^7 cells/ml. Twenty % NaCl solution was used for cell washing and resuspension.

Thiobacillus intermedius ATCC 15466 and Escherichia coli B/r were also kindly supplied by Dr. T. Morinaga and Dr. S. Kondo, respectively. To grow these bacteria, Luria Bertani medium (LB) was used, containing 10 g NaCl, 10 g polypeptone and 5 g yeast extract (Difco) per liter of distilled water, adjusting to pH 6.8. Cells of T. intermedius and E. coli B/r were grown up at 33°C for 12 h and 37°C for 3 h, respectively. Cells at logarithmic phase were collected by centrifugation, washed, and then resuspended by the same way as mentioned above. Phosphate buffer (67 mM, pH 6.8) was used for cell washing and resuspension.

γ-Irradiation

The cell suspension (3 ml) was irradiated in a 5 ml glass tube with ^{60}Co γ-rays at a dose-rate of 3 Gy/min at 0°C. Colony counting was performed on agar plates after incubation at 37°C for 7-8 days (H. salinarium) and for over night (E. coli B/r), and at 33°C for 3-4 days (T. intermedius). For preparation of agar medium, 1.5% agar was added to each medium.

UV-Irradiation

The cell suspension (5 ml) was irradiated in a petri dish (ϕ 5 cm) at a distance of 40 cm from a Mitsubishi Electric 15-watt germicidal lamp, having an intensity of 0.4 J/m^2/sec. The suspension was stirred with a magnetic stirrer during exposure. Experiments were done under yellow light. Colony counting on the agar plate was performed by the same way as for γ-irradiation.

Mitomycin C Treatment

Mitomycin C solutions (100 µg/ml) were prepared for each set of experiments by dissolving mitomycin C in phosphate buffer (67 mM, pH 6.8) for T. intermedius and E. coli B/r or 20% NaCl for H. salinarium and filter-sterilized. Different small volumes of this solution were added to 10 ml of the cell-suspended solutions (10^7 cells/ml) in light-proof tubes (since mitomycin C is unstable under light). These tubes were left standing for 1 h in an ice-water bath, centrifuged, washed with phosphate buffer or 20% NaCl three times to remove mitomycin C, and then resuspended in 10 ml of the buffer or NaCl solution. Colony counting on the agar
plate was performed by the same way as for γ-irradiation. To measure the incorporation of mitomycin C into cells, the supernatant obtained after centrifugation of treated cells was directly subjected to spectrophotometric measurement at 363 nm\(^\text{14}\). The amount of mitomycin C uptake was calculated from the difference between initial and final (1h incubation) concentrations of mitomycin C in the media.

**RESULTS**

Figure 1 shows survival curves of *H. salinarium*, *T. intermedius*, and *E. coli B/r* by irradiation with \(^{60}\text{Co}\) γ-rays. Their D\(_{37}\) values were 393 Gy, 150 Gy, and 92 Gy (90 Gy reported by Sweet et al.\(^\text{15}\)), respectively. D\(_{37}\) of *E. coli H/r30*, which is another radioresistant mutant, was reported to be 50 Gy by irradiation with X-rays\(^\text{16}\). *T. intermedius* was more resistant than these two radioresistant mutants of *E. coli*, while *H. salinarium* was much more resistant than *T. intermedius*.

Figure 2 shows survival curves of the three bacteria by irradiation with UV light. Their D\(_{37}\) values were 212 J/m\(^2\), 38 J/m\(^2\), and 10 J/m\(^2\), respectively. It was reported that D\(_{37}\) of *E. coli H/r30* was 15 J/m\(^2\) \(^\text{16}\) which is similar to that of *E. coli B/r*. *T. intermedius* was resistant than these radioresistant mutants of *E. coli*, while *H. salinarium* was much more resistant than *T. intermedius*. This tendency was the same as the case of the ionizing radiation described above.

Figure 3 shows survival curves of the three bacteria by exposure to mitomycin C. Their D\(_{37}\) values were 2.36 µg/ml/h, 0.25 µg/ml/h, and 0.53 µg/ml/h, respectively. It was reported that D\(_{37}\) of *E. coli H/r30* was 0.5 µg/ml/h\(^\text{16}\) which is almost the same as that of *E. coli B/r*. In the case of mitomycin C treatment, however, these *E. coli* strains were more resistant than *T. intermedius*, differing from the cases of exposures...
Fig. 2. Survival curves of *Halobacterium salinarium* (■), *Thiobacillus intermedius* (●), and *Escherichia coli* B/r (▲) after exposure to UV light compared with that of *Escherichia coli* H/r30 (dashed line). The mean of surviving fractions and their standard deviations were based on five independent experiments.

Fig. 3. Survival curves of *Halobacterium salinarium* (■), *Thiobacillus intermedius* (●), and *Escherichia coli* B/r (▲) after exposure to mitomycin C compared with that of *Escherichia coli* H/r30 (dashed line). The mean of surviving fractions and their standard deviations were based on five independent experiments.
to ionizing radiation and UV light, though *H. salinarium* was much more resistant than these *E. coli*. The amount of mitomycin C incorporated into bacterial cells was determined by measuring the concentration of mitomycin C remaining in the media. The results are summarized in Table 1 and indicate that the incorporation into *H. salinarium* is about five times as much as those into *T. intermedius* and *E. coli B/r*.

### Table 1. Incorporation of mitomycin C (MMC) calculated from absorbance at 363 nm of the media after eliminating cells.

<table>
<thead>
<tr>
<th>MMC (µg/ml)</th>
<th><em>H. salinarium</em> (µg/10⁶ cells)</th>
<th><em>T. intermedius</em> (µg/10⁶ cells)</th>
<th><em>E. coli B/r</em> (µg/10⁹ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>6.77±1.24</td>
<td>ND</td>
<td>0.68±0.90</td>
</tr>
<tr>
<td>[0.266±0.007]</td>
<td>[0.206±0.008]</td>
<td>[0.260±0.003]</td>
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</tr>
<tr>
<td>2.0</td>
<td>5.27±0.66</td>
<td>1.10±0.44</td>
<td>0.99±0.55</td>
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<tr>
<td>[0.182±0.003]</td>
<td>[0.134±0.005]</td>
<td>[0.172±0.002]</td>
<td>[0.173±0.004]</td>
</tr>
<tr>
<td>1.0</td>
<td>2.37±0.75</td>
<td>0.96±0.65</td>
<td>0.54±0.22</td>
</tr>
<tr>
<td>[0.093±0.002]</td>
<td>[0.071±0.007]</td>
<td>[0.084±0.006]</td>
<td>[0.088±0.002]</td>
</tr>
<tr>
<td>0.5</td>
<td>ND</td>
<td>0.53±0.53</td>
<td>0.11±0.53</td>
</tr>
<tr>
<td>[0.047±0.001]</td>
<td>[0.042±0.005]</td>
<td>[0.046±0.005]</td>
<td></td>
</tr>
</tbody>
</table>

* Data show the mean values of three separate experiments with standard deviations.

### DISCUSSION

The results obtained in the present study are summarized in Table 2. It is apparent from the data that the resistibility to the lethal effects of γ-rays, UV light, and mitomycin C of *H. salinarium* is greater than that of *T. intermedius* and *E. coli B/r*.

To the lethal effect of γ-rays, the resistibility factors of *H. salinarium* in comparison with *T. intermedius* and *E. coli B/r* were 2.6 and 4.3, respectively, and the factor of *T. intermedius* to *E. coli B/r* was 1.6. Why is

### Table 2. D_{37} values and relative resistibility to γ-rays, UV light, and mitomycin C (MMC) for *Halobacterium salinarium* (H), *Thiobacillus intermedius* (T), and *Escherichia coli B/r* (E).

<table>
<thead>
<tr>
<th>Lethal Source</th>
<th>D_{37} *</th>
<th>Relative Resistibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>T</td>
</tr>
<tr>
<td>γ-rays (Gy)</td>
<td>393</td>
<td>150</td>
</tr>
<tr>
<td>UV light (J/m²)</td>
<td>212</td>
<td>38</td>
</tr>
<tr>
<td>MMC (µg/ml/h)</td>
<td>2.36</td>
<td>0.25</td>
</tr>
</tbody>
</table>

* Dose required to reduce viability to 37%.

* Dose unit for the data.
**H. salinarium** much more resistant than *T. intermedius* and *E. coli B/r*? Very concentrated NaCl solution was used as the medium of *H. salinarium*. However, the high resistibility of *H. salinarium* is not due to the salt effect[7]. Previously, Saito *et al.* have demonstrated that an extremely radioresistant bacterium, *Rubrobacter radiotolerans*, has bacterioruberin as the main pigment[8], which has highly conjugated double bonds (F18) and is a very efficient scavenger of OH radicals[9]. This pigment was also found in Halobacteria[10], though the content was less than that in *R. radiotolerans*. Indeed, the pigmentless mutants were found to be more radiation sensitive than the wild type in some radioresistant bacteria[18,19]. Thus, the high resistibility of *H. salinarium* to γ-irradiation may be partly attributable to the presence of bacterioruberin. *T. intermedius* was more resistant than *E. coli B/r*. The resistibility of *T. intermedius* may be due to the higher sulfur content because, like bacterioruberin, sulfur-containing compounds are effective free radical scavengers[10]. In addition, other factors, such as the differences in DNA repair capacities, might be also involved in the higher resistibility of *H. salinarium* and *T. intermedius* than *E. coli B/r*. Concerning this, the inducible repair capacities by hydrogen peroxide enhance the survival of *E. coli* after exposure to X-rays, but not to UV and mitomycin C[20]. These findings may also have some relations with the resistibility differences to UV and mitomycin C among the bacteria as discussed below.

To the lethal effect of UV light, the resistibility factors of *H. salinarium* in comparison with *T. intermedius* and *E. coli B/r* were 5.6 and 21.2, respectively, and the factor of *T. intermedius* to *E. coli B/r* was 3.8. These factors are much higher than those in the case of γ-irradiation. The photoreactivation and the lack of dark excision repair are well known in Halobacteria[5-9]. This experiment, however, was performed under yellow light to avoid photoreactivation. Therefore, the present results suggest the several possibilities that bacterioruberin in *H. salinarium* cells strongly scavenges active oxygen species or acts as an UV energy absorber and that some special enzymes exist for repair of pyrimidine dimers. The reason should be thoroughly investigated in future.

To the lethal effect of mitomycin C, the resistibility factors of *H. salinarium* in comparison with *T. intermedius* and *E. coli B/r* were 9.4 and 4.5, respectively. The value of 9.4 in mitomycin C treatment was much higher than the value of 2.6 in γ-irradiation. Furthermore, the incorporation of mitomycin C into *H. salinarium* cells was about five times higher than those into *T. intermedius* and *E. coli B/r* cells (Table 1). Considering these incorporated amounts, *H. salinarium* has an even higher resistibility than *T. intermedius* and *E. coli B/r*. These results suggest that *H. salinarium*, but not *E. coli*, might have specific enzymes for inactivation of mitomycin C or repairing of DNA damages produced by mitomycin C. On the other hand, the resistibility factor of *T. intermedius* to *E. coli B/r* was 0.5. The higher resistibility of *T. intermedius* over *E. coli B/r* to ionizing radiation and UV light but not to mitomycin C suggests that the larger amount of sulfur compounds in *T. intermedius* protects cells against lethal damages by ionizing radiation and UV light, but not those by mitomycin C.

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REFERENCES


