INDUCTION OF LYSOGENIC MICROCOCCUS LYSODEIKTICUS

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ABSTRACT

Field, A. K. (Cornell University, Ithaca, N.Y.) and H. B. Naylor. Induction of lysogenic Micrococcus lysodeikticus. J. Bacteriol. 84:1129-1133. 1962.—Between 50 and 60% of log-phase cells of Micrococcus lysodeikticus strain ML 53-40, lysogenic for N5 bacteriophage, were induced by an optimal dose of ultraviolet light. Induction of dilute cell suspensions caused a subsequent 200- to 800-fold increase in infective-center concentration. Cells in the stationary growth phase and cells adapted to a chemically defined medium were not induced by ultraviolet irradiation. Dimethyl sulfate induced the lysogenic culture to about the same extent as did ultraviolet light, whereas β-propiolactone was less effective.

Lwoff, Siminovitch, and Kjeldgaard (1950) reported that the exposure of lysogenic Bacillus megaterium 899 cells to ultraviolet light caused subsequent lysis and release of bacteriophage particles by nearly every cell. Ultraviolet irradiation was also shown to be a highly effective inducing agent for both Escherichia coli K-12 (Weigle and Delbrück, 1951) and Pseudomonas pyocyanea (P. aeruginosa; Jacob, 1952). On the other hand, Groman, Eaton, and Booker (1958) found that for Corynebacterium diphtheriae strain C4, lysogenic for either phage γ or β, the optimal dose of ultraviolet irradiation induced only 10 to 15% of the bacterial population.

Many chemicals have proven to be effective inducing agents. Williams Smith (1953) demonstrated that cells of lysogenic Salmonella ser. thompson were induced when grown in the presence of sodium thiolactate, glutathione, or sulfathiazole. The antibiotics L-azaserine, mitomycin C, and phageolissin A58 have also been reported to possess inducing ability in certain lysogenic systems (Gots, Bird, and Mudd, 1955; Otsuji et al., 1959; Hall-Asheshov and Asheshov, 1956).

In this study, various physical and chemical agents were tested for ability to induce bacteriophage production by a lysogenic culture of Micrococcus lysodeikticus (Fleming, 1922).

MATERIALS AND METHODS

Bacteria and bacteriophage cultures. A pink-pigmented mutant of M. lysodeikticus (ML 53-5) and ML 53-40, a culture of ML 53-5 artificially lysogenized with bacteriophage N5, were obtained from the Cornell Culture Collection. N5 bacteriophage was originally isolated from sewage, by use of M. lysodeikticus as host.

The lysogenic nature of ML 53-40 was verified by repeatedly restreaking from isolated colonies. From the final streaked plate, a single colony was picked and inoculated into broth. A suitable dilution of the resultant culture was plated by the soft-agar technique to obtain well-isolated colonies. After incubation at 30°C for 24 hr, at which time colonies were just visible macroscopically, the plates were thoroughly seeded with the indicator strain, ML 53-5. Seeding was accomplished by exposing the surface of the agar to an aerosol produced with a DeVilbiss nebulizer. Upon further incubation, each ML 53-40 colony produced a clear zone in the indicator lawn, and N5 phage could be recovered from these zones. The preincubation procedure was adopted after finding that addition of indicator cells simultaneously with the lysogenic cells resulted in formation of only approximately 10% of the calculated number of plaques, and no centered colonies could be observed in these plaques. The lysogenic cells were immune to N5 bacteriophage attack from the outside.

Culture media. Broth used in daily transfers consisted of 1.0% peptone, 0.5% yeast extract, 0.1% glucose, and 0.5% NaCl. The pH was adjusted to 7.5 before autoclaving at 121°C for 15 min. Solid semisolid agar contained the same constituents as transfer broth, with the addition

1 Taken in part from a dissertation submitted by the senior author to the Graduate Faculty of Cornell University, in partial fulfillment of the requirements for the degree of Master of Science.

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of 1.5 and 0.7% agar, respectively. The composition of the chemically defined media was as described by Wolin and Naylor (1957). Gelatin-phosphate diluent used in titering bacteria and bacteriophage contained 0.2% gelatin, 0.7% K$_2$HPO$_4$, and 0.3% KH$_2$PO$_4$.

Assay of bacteria and bacteriophage. Bacteria titers were determined by spreading 0.1-ml samples of the appropriate dilution on the surface of solid agar and incubating them at 30°C for a minimum of 48 hr. Bacteriophage assays were determined by a modified Gratia (1936) technique; 0.3 ml of a young turbid culture of indicator bacteria, 0.1 ml of the phage suspension, and 4 ml of melted and tempered semisolid agar were pipetted directly onto a hard-agar surface. After thorough mixing, the agar was allowed to gel, and the plates were incubated at 30°C for a minimum of 24 hr before counting the plaques.

Inactivation of bacteria and bacteriophage with ultraviolet light. Broth inoculated with 20% of a 24-hr culture was vibrated in a 9-kc Raytheon Sonic Oscillator for 5 min at room temperature. This treatment reduced cell clumps to primarily single and paired cells, with no apparent effect on viability. After suitable incubation to allow the cells to begin multiplying, the culture was diluted to $10^5$ cells/ml in gelatin-phosphate diluent. An open 9-cm petri plate containing 5 ml of cell suspension was agitated constantly while being exposed to ultraviolet rays from a 15-w General Electric Germicidal Lamp at a distance of 63 cm. Bacterial assays were made on 0.1-ml samples taken after various periods of exposure. All manipulations were performed under subdued yellow light, and the plates were incubated in the dark.

A bacteria-free N5 lysate was diluted with gelatin-phosphate diluent to $10^2$ phage/ml for irradiation. All subsequent steps were carried out as indicated for bacterial irradiation studies.

Determination of optimal ultraviolet induction dose. Lysogenic cells were prepared and irradiated for increasing time intervals, as described for ultraviolet inactivation of bacterial cells. After each exposure period, a 0.1-ml sample was diluted 1:100 in fresh transfer broth, incubated on the reciprocal shaker at 30°C for 4 hr, and assayed for infective centers.

Determination of latent periods following induction. All determinations of latent and rise periods were made by a modification of the single-step growth technique described by Adams (1959). Lysogenic cells were prepared as described for ultraviolet inactivation of bacterial cells. Before and after irradiation at the optimal dose, samples were diluted 1:100 with fresh transfer broth, and incubated at 30°C on the reciprocal shaker. Infective-center assays were made at intervals during the incubation period.

Induction with agents other than ultraviolet light. A log-phase culture ($10^9$ cells/ml) was incubated with the chemical agent under study for 30 min, after which the suspension was diluted to $10^9$ cells/ml with transfer broth, and incubated for 4 hr before assaying for infective centers. Tests for induction by thermal shock were performed by exposing vials containing 1 ml of cell suspension ($10^9$ cells) to 55°C, in a water bath, for increasing time intervals.

RESULTS

Induction with ultraviolet light. Susceptibility of lysogenic M. lysodeikticus to induction by ultraviolet light was first indicated when the ultraviolet light killing curves for ML 53-5 and ML...
53-40 were compared. As shown in Fig. 1, the surviving fraction of ML 53-40 decreased much more rapidly than that of ML 53-5, with increasing doses of irradiation. (For comparison, the inactivation rate for free N5 particles is also shown.) Since ML 53-5 and ML 53-40 differ only in that the latter is lysogenic, it was suspected that the difference in survival was due in part to induction of lysogenic cells. To obtain direct evidence on this point, experiments were conducted with ML 53-40 cells washed twice just prior to preparing the diluted suspension for exposure to ultraviolet light. Washing was necessary to reduce the free phage content of the final suspension to a negligible level (approximately 50/ml). The suspension was then exposed to ultraviolet light, and samples were taken at intervals. Appropriate dilutions were prepared, and 0.1-ml portions were spread on hard-agar surfaces. Counts of cells capable of producing colonies were made after incubating the plates for 48 hr in the dark. The surface of each plate was then "fogged" with ML 53-5 cells by the procedure described under Materials and Methods. After an additional incubation for 24 hr, plaques without centered colonies were counted. Each plaque was assumed to have been initiated by the phage particles remaining on the agar after lysis of a single induced cell. Results of a typical experiment are recorded in Table 1. Experiments performed on different batches of cells under the described conditions showed that maximal induction varied between 50 and 60% with exposure times of 4 to 6 min.

### Table 1. Comparison of killing and inducing effects of ultraviolet light on ML 53-40

<table>
<thead>
<tr>
<th>Exposure time</th>
<th>Colony formers (x 10⁶)</th>
<th>Survival</th>
<th>Clear plaques* (x 10⁶)</th>
<th>Induced</th>
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<tbody>
<tr>
<td>min</td>
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<td>%</td>
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<tr>
<td>6</td>
<td>4</td>
<td>5</td>
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* Induced cells.

**FIG. 2. Induction of ML 53-40 with ultraviolet light: □, ML 53-40 (log-phase cells); ○, ML 53-40 (stationary-phase cells); Δ, ML 53-40 (adapted to chemically defined medium).**

**FIG. 3. Development of infective centers after ultraviolet treatment: □, ML 53-40 (log-phase cells); ○, ML 53-40 (stationary-phase cells); Δ, ML 53-40 (adapted to chemically defined medium); 1 = 4 min of irradiation; 2 = 0 min of irradiation.**
The optimal dose of ultraviolet light for maximal production of phage by ML 53-40 was determined by the procedure outlined under Materials and Methods. Exposure of the cells to irradiation resulted in marked increases in numbers of infective centers, up to the 4-min point (Fig. 2). Longer exposures resulted in decreased numbers. Susceptibility to induction with this agent was essentially the same for cells cultured on the chemically defined medium as for cells produced on transfer broth; however, the yield of infective centers was usually greater with the latter. In either case, the yield varied considerably with different experiments.

Latent and rise periods after ultraviolet treatment of ML 53-40 were determined for both log-phase and stationary-phase cells grown in transfer broth, and for cells adapted to the defined medium. To eliminate variations due to differences in initial cell concentrations, increases in numbers of infective centers relative to the number present in nonirradiated controls at the start of the incubation period were plotted against postirradiation incubation time. The number of infective centers produced in the nonirradiated cultures of log-phase cells propagated either on transfer broth or chemically defined medium increased 1.4-fold per hr of incubation (Fig. 3). This rate of increase also occurred with stationary-phase cells after a lag period of 1 hr following dilution in fresh broth. After irradiation for 4 min, log-phase cells from transfer broth underwent a 1- to 2-hr latent period, followed by a 2-hr rise period during which the infective-center concentration increased about 800-fold. Similar treatment of stationary-phase cells resulted in a 1-hr extension of the latent period followed by a 400- to 500-fold increase in infective-center concentration. Cells of ML 53-40 adapted to chemically defined medium also had a latent period of about 3 hr, but the final level of infective centers was much lower than that obtained with induced cells from complex broth.

**Induction of ML 53-40 with other agents.**

β-Propiolactone and dimethyl sulfate were both found to have an inducing effect on ML 53-40. Preliminary experiments showed that, at equal concentrations, these two agents had comparable killing effects on ML 53-4. Concentrations of 0.05 and 0.1% of either agent killed, respectively, 5 and 20% of the cells in 30 min. Although their killing effects were comparable, there was a quantitative difference in their inducing effects. The maximal increase in infective centers resulted from treatment of cells with approximately 0.05% of either agent (Fig. 4). The actual increase, however, was about ten times greater with dimethyl sulfate than with β-propiolactone.

Other agents tested and found to give negative results with ML 53-40 under our conditions were H₂O₂, sulfathiazole, reduced glutathione, and thermal shock.

**DISCUSSION**

The above evidence indicates that log-phase cells of ML 53-40 grown in complex broth are inducible with ultraviolet light. Whereas the percentage of the lysogenic cell population induced increased with increasing doses of irradiation, the fraction of the total population that remained viable diminished. Consequently, under our experimental conditions the optimal irradiation dose for maximal phage production occurred with a 4-min exposure period.

Lwoff et al. (1950) found that cells of *B. megaterium* in the stationary phase of growth on a complex medium or in the log phase of growth on a defined medium were more refractory to in-
duction by ultraviolet irradiation than were log-
phase cells on a complex medium. Comparison of
inducibility of ML 53-40 from log and stationary
growth phases on complex medium suggested that
there was a slight reduction in the fraction of cells
induced in stationary phase. Also, those cells that
were induced underwent an extended latent
period. Since there was a lag in the production
of infective centers by nonirradiated stationary-
phase cells after being transferred to fresh broth,
the extended latent period of induced cells was
probably due to a similar period of adaptation to
the fresh medium. Similarly, ML 53-40 adapted
to defined medium remained inducible, but with
a slightly reduced infective-center increase,
compared with that produced by induced cells
grown on transfer broth.

Jacob and Wollman (1953) suggested that a
relation exists between mutagenic, carcinogenic,
and inducing agents. Known mutagens, such as
nitrogen mustard, mustard gas, and ultraviolet
light, were established as mutagens as well as in-
ducing agents. Our limited observations support
this relationship. Smith and Srb (1951) found that
β-propiolactone caused reversions to growth-
factor independence in biochemical mutants of
Neurospora crassa. As shown in this study,
β-propiolactone can also function as an inducing
agent for the development of bacteriophage by
lysogenic M. lysodeikticus. Dimethyl sulfate, an
active methylating agent, was used by Kölmark
(1956) to induce mutations to independence in
adenineless and inositol-less strains of N. crassa.
In the present study, it proved to be an effective
inducing agent for bacteriophage production as
well.

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