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Paper Replication Method for Isolation of Radiation-Sensitive Mutants†

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A filter paper replication system particularly useful for isolation of radiation-sensitive mutants of pigmented bacteria was devised. The fidelity of replication was high. Adhesion between a paper disk and a properly dried master plate provided adequate contact pressure. The replicas arising from this technique constitute a convenient apparatus for general application in isolation of clones sensitive to a discriminating treatment.

The replica plating technique of Lederberg and Lederberg (1) is widely used for mass transfer and isolation of unique biotypes with few limitations (2). The technique is impractical, however, where there is a need for a treatment such as irradiation of cells on the replicator (L. C. Keller, Ph.D. dissertation, University of Nebraska, Lincoln, 1981). When the discriminatory treatment is for selection of sensitive cells, the number of cells in each transfer unit is important. Discrimination may be due to relative susceptibility or to differences in cell numbers in the transfer units. The latter situation is likely to happen if the replication operation is poor in reliability, i.e., the replicator used is unable to (i) pick up a similar number of cells from every colony it contacts or (ii) transfer a similar number of cells from every colony it has picked up to an agar plate, which is to be irradiated for discrimination. A replica plating technique is considered ideal for isolation of radiation-sensitive mutants if it is able to discriminate between deletion of colonies of sensitive cells and deletion of colonies with a low population density.

In our hands, the Lederberg technique failed to transfer uniform numbers of cells from individual colonies. As a result, large numbers of false-positive radiation-sensitive colonies arose. Furthermore, the velveteen pads were not practical carriers for discriminatory treatments.

A simple, reliable replica plating method particularly useful for isolation of radiation-sensitive mutant strains of bacteria was developed. This method used filter paper disks as replica-

MATERIALS AND METHODS

Organism and culture conditions. A pink-to-red micrococcus (4) was propagated on plate count agar (Difco Laboratories) at 32°C and stored at 5°C. Cultures were rejuvenated in m-Plate Count Broth (Difco Laboratories) in a shaker incubator at 32°C.

Replicator paper. Whatman no. 1 filter paper (9 cm in diameter) was cut as shown in Fig. 1. The protruding part of the filter paper was folded up to facilitate movement with forceps. About 25 filter paper disks were stacked in each of the glass petri dishes, which were wrapped with aluminum foil to avoid condensation of water. The packages were then autoclaved at 121°C for 15 min.

Preparation of agar plates. Plates of plate count agar were prepared according to methods outlined by Speck (3). For properly prepared, dried plates, the following procedure was used. After pouring, the agar was allowed to solidify and dry overnight at room temperature. Then the plates were stacked to save space, and the drying process was continued at room temperature for 2 to 3 days depending on the humidity of the surrounding air. When more rapid drying was needed, plates were stored at 48°C with the lids partly removed. An easy way to check for proper dryness was to drop 0.01 ml of distilled water onto the agar of a test plate. The dryness was considered acceptable if the water was absorbed within 20 to 40 min. An experienced operator readily recognizes the constraints on drying.

Preparation of master plates and replication. Log-phase cells were suitably diluted, spread on plate count agar (about 100 CFU per plate), and incubated for 17 to 18 h until barely visible colonies were formed. For replication, a filter paper was placed on a master plate surface to give a single point contact and then allowed to fall into the surface where adhesion established nonshifting contact. Also, the filter paper was transferred so that the protruding part of the paper matched a reference mark placed on the side of each petri dish bottom. After 1.5 min of contact, which allowed the paper to become saturated with liquid, the paper was transferred with the contact side up to a sterile petri dish. The same master plate was then

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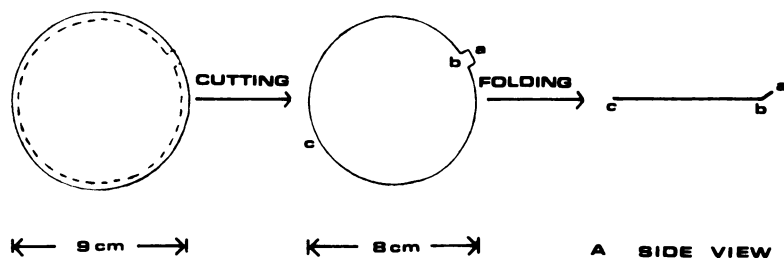


FIG. 1. Preparation of a paper replicator.

rereplicated with another filter paper disk. After replication, the master plate was incubated overnight at 32°C and then stored at 5°C for further reference. The filter paper replicas (FPRs) were available for discriminating treatment of the replica colonies (RCs).

Pretreatment of RCs for discrimination and viability tests. Some FPRs were used immediately after replication to observe the behavior of log-phase cells in the invisible RCs. When the discriminating treatment involved a study of mature RCs, the FPRs were preincubated at 32°C for 1 to 2 days. Nutrients and water absorbed during the replication step allowed the RCs to grow to visible size (0.5 to 1.0 mm in diameter). These two preconditionings determined how the discrimination-treated RCs were tested for viability. If the RCs were invisible at the time of discrimination treatment, the viability tests were done by incubating the treated RCs in a humid environment, e.g., over distilled water in a desiccator. For RCs pregrown to visible size, the viability test was conducted by incubating each FPR on a fresh agar medium to allow surviving RCs to grow.

Irradiation as the discriminating treatment. UV irradiation was carried out at room temperature with a General Electric G15T8 germicidal lamp at a distance of 33 cm from the FPRs, giving an incident dose rate of

$20 \text{ J m}^{-2} \text{ s}^{-1}$ as measured with a YSI-Kettering radiometer (model 65A). For gamma irradiation, the FPRs were irradiated at $30 \pm 10^\circ\text{C}$ with a ^{60}Co source at a dose rate of approximately 5 krad/min.

Radiation resistance of RCs. Radiation resistance of RCs was expressed as the dose required to inactivate 50% of the RCs on an FPR (LD_{50}). LD_{50} was estimated by plotting the percentages of the surviving RCs against doses of irradiation. The percentage of surviving RCs was calculated by dividing the number of viable RCs on an FPR by the total number of colonies in the paper-contacted area on the corresponding master plate and then multiplying by 100.

RESULTS

Reliability of the paper replication method. To evaluate the performance of the paper replication method, master plates and their FPRs were incubated at 32°C for 2 days. Typical results demonstrating the relationship between a master plate and its two FPRs are shown in Fig. 2. The RCs appeared to grow within sharp boundaries, whereas colonies on the master plate showed signs of slight tailing. However, the tailing posed

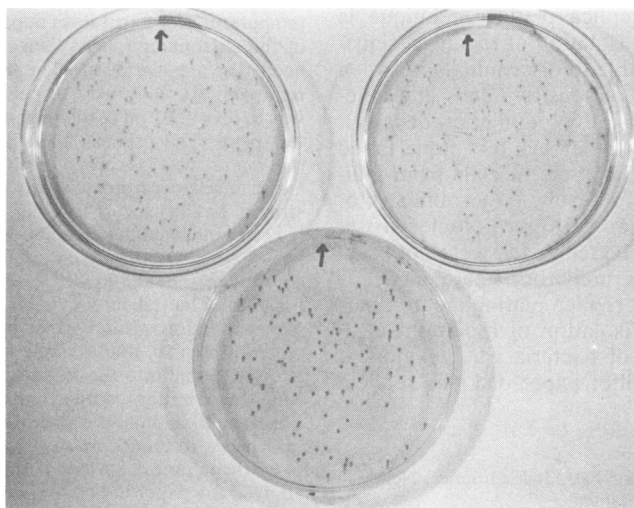


FIG. 2. A master plate (bottom) and its two filter paper replications.

no difficulty in isolation of a colony. The fidelity of replication was 100%. Spurious colonies were not found on any of the FPRs.

To determine the quantitative reliability of the method, the kinetics of radiation destruction of the RCs were determined. Six or more plates and their FPRs from a single culture were prepared for each irradiation experiment. Either UV or gamma irradiation was used for RCs containing log-phase cells; only gamma radiation was applied to RCs containing mature cells. The FPRs containing the log-phase cells were treated in individual sterile petri dishes. The FPRs containing the mature cells were irradiated in groups of five separated by sterile aluminum foil. After exposure to the proper dose of either UV or gamma irradiation, each FPR was incubated for estimation of the number of surviving RCs, according to the procedures previously described. Survival curves based on three replications are shown in Fig. 3. All curves are marked by a precipitous decline where inactivation occurred. This implies that RCs from the same culture were satisfactorily homogeneous with regard to radiation resistance. This, in turn, suggests that the filter paper replicators reliably picked up a uniform portion of cells from each colony contacted.

Radiation as the discriminating agent for sensitive RCs. An estimation of the proper radiation dose was obtained from the results presented in Fig. 3. The LD₅₀ for various conditions was estimated to be 5.4×10^4 J/m² when UV irradiated immediately after replication, 630 krad when gamma irradiated immediately after replication, and 5.2 Mrad when gamma irradiated after receiving an incubation/drying treatment. Based on the LD₅₀, proper dose and conditions of irradiation for screening RCs for radiation-sensitive mutants of the test micrococcus could be determined, e.g., $\frac{1}{5}$ to $\frac{1}{10}$ of the LD₅₀ would be considered appropriate.

DISCUSSION

Radiation resistance of a colony is a function not only of the resistance of the cells present but also of the number of cells it contains. Therefore, all colonies on a master plate derived from a single culture should behave similarly in response to a discriminating treatment. The RCs should also have the same population, provided the replication had been quantitative. The paper replication method showed a very low variability because the RCs prepared from a single culture were uniformly radiation resistant. This was evidenced by the radiation survival curves, which showed a precipitous inactivation phase (Fig. 3).

The paper replication method is well suited for isolation of radiation-sensitive mutants of pig-

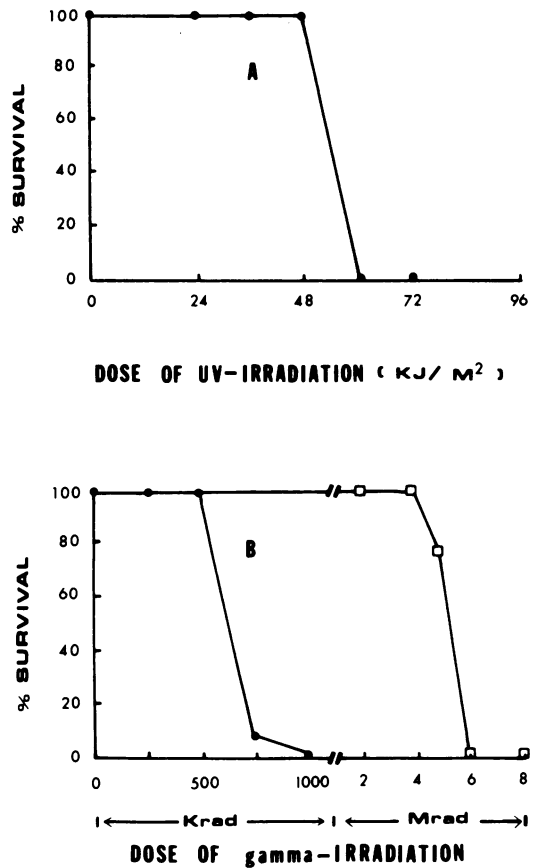


FIG. 3. Survival curves of the RCs exposed to UV (A) or gamma (B) irradiation. Symbols: ●, FPRs without incubation/drying pretreatment; □, FPRs with incubation/drying pretreatment.

mented cultures. It is possible to irradiate the RCs under various environmental conditions. This flexibility should expedite work on the mechanism of radiation resistance. In our laboratory, this paper replication system has been successfully used in the isolation of radiation-sensitive mutants of a highly radiation-resistant micrococcus after an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis treatment (manuscript in preparation).

The paper replication method has potential in that the filter paper disks carry the RCs through subsequent procedures for discrimination. For example, the FPRs might be a convenient apparatus for discriminating colonies for susceptibility to agents such as gases and heat. Effects of physiological ages of cells or colonies can also be studied by preincubating the FPRs for a proper period of time before the treatments are given. When there is a lack of pigment for contrast between the RCs and the filter paper,

physiological reaction of the RCs may be used as a criterion of viability. For example, growth in the presence of 2,3,5-triphenyltetrazolium chloride and the formation of red colonies could be used for many bacteria (3).

The uniqueness of the method is the fidelity of establishing contact between the paper and the surface of the master plate. Once contact is made between the filter paper and the properly dried agar, they are drawn together. Adhesion through absorption then maintains uniform, stable contact. Compared to the commonly used velveteen cloths, the filter paper disks are superior in surface quality. The simplicity of the

method should make it useful for many purposes.

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