JOURNAL OF VIROLOGY, July 1968, p. 710-715
Vol. 2, No. 7

Printed in U.S.A.

Mechanisms of Enhancement of SP82 Transfection[1]

H. T. EPSTEIN AND INGA MAHLER

Department of Biology and Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

Received for publication 8 March 1968

SP82 transfection in *Bacillus subtilis* could be markedly increased by exposing the competent cells to ultraviolet (UV)-irradiated homologous or heterologous cellular deoxyribonucleic acid (DNA). This enhancement was similar in time and level of peak effect to enhancements effected by preinfection with helper phages or by UV irradiation of competent cells. The effectiveness of various DNA preparations in increasing transfection paralleled the adenine plus thymidine content of the preparations and was maximal at UV doses approaching those which were maximal for pyrimidine dimerization. The most probable interpretation is that irradiated DNA binds cellular nucleases which would otherwise inactivate the incoming transfecting DNA.

Transfection by SP82 phage deoxyribonucleic acid (DNA) in *Bacillus subtilis* can be enhanced by two different means. Green (6) showed an enhancement resulting from adding helper phages before or after the addition of transfecting DNA. In addition to enhancing the transfection, the formation of plaques was changed from an approximately fourth-power dependence on DNA concentration to an approximately first-power dependence.

Recently, Epstein (4) showed an enhancement resulting from ultraviolet (UV) irradiation of competent cells of *B. subtilis* before addition of SP82 DNA. This enhancement is similar to that described by Green both in increase of plaque formation and in effecting a first-power dependence on DNA concentration.

Neither enhancement method raises the maximal concentration of plaque-forming cells above the saturation level produced by a particular preparation of DNA. Thus, these methods do not seem to create new competent cells but rather to decrease the factors which interfere with plaque formation by transfecting DNA in some of the existing competent cells.

This paper reports the results of studies designed to increase our understanding of the enhancement mechanisms and describes an additional method for increasing SP82 transfection.

MATERIALS AND METHODS

SP82 lysates were made by inoculating 1 liter of vigorously shaking *B. subtilis* strain SB-1 cells (10^8/ml) with a phage multiplicity of 0.1. The lysate was adjusted to be 0.005 M $MgSO_4$, and 1 to 2 μg/ml each of deoxyribonuclease and ribonuclease were added for 1 hr at 37 C. A 900-ml amount of the lysate was centrifuged to obtain phage pellets which dissolved overnight in 5 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate). After clarification by low-speed centrifugation (12,000 $\times$ *g* for 10 min), the preparation was used as the starting material from which transfecting DNA was extracted as previously described (4). The sedimentation properties of SP82 transfecting DNA, as determined by neutral sucrose gradient and by analytical ultracentrifugation, were similar to those reported by Green (6, 7). The remainder of the phage lysate was used for viable phage experiments. As the phage concentration was about 2×10^{10}/ml, the dilution involved in the various experiments lowered the residual nuclease concentrations below those which might have a detectable effect on extracellular nucleic acids during the incubation times used.

Preparation of competent cells of *B. subtilis* strain SB-1 (histidine$^-$, tryptophan$^-$) began with overnight growth of bacteria on Tryptose Blood Agar Base (Difco). Precompetent cells were produced by scraping the cells grown overnight off the agar and allowing them to grow for 4 hr in growth medium (minimal medium of reference 1 plus 0.5% glucose, 0.02% casein hydrolysate, 50 μg of L-tryptophan, 5×10^{-3} M $MgSO_4$, and 0.04% histidine). Cells were made competent by 10-fold dilution and 90 min of growth in competency medium (minimal medium plus 0.5% glucose, 0.01% casein hydrolysate, 5 μg of L-tryptophan, and 5×10^{-3} M $MgSO_4$). Precompetent cells could be preserved with full activity for weeks by adding 15% glycerol or 5% dimethylsulfoxide and rapid freezing in a dry ice-acetone bath; the cells were then stored in a freezer at -60 C.

Two different germicidal lamps were used for UV irradiation. A standard General Electric 15-w germi-

[1] Contribution no. 583 from the Graduate Department of Biochemistry.

cidal lamp gave a dose rate of about 70 ergs/mm² per sec to samples 17 cm from the lamp. A General Electric G4T4 germicidal lamp produced about 7 ergs/mm² per sec at the 30-cm sample distance.

Transfection procedures and materials were those described by Green (6), with minor modifications described by Epstein (4). Cellular DNA was isolated by the method of Marmur (9), except for the DNA of *Dictyostelium discoideum* which was the kind gift of Racquel Sussman.

Unless otherwise stated, the following procedure was used for transfections. Frozen precompetent cells of *B. subtilis* strain SB-1 were thawed, diluted 10-fold in competency medium, and shaken vigorously at 37 C for 90 min. The cell concentration increased from 2×10^8 to 8×10^8 cells/ml. Samples (0.5 ml) were distributed into chilled test tubes, and 1 μg (in 0.1 ml) of transfecting DNA was carefully added to the cells. After 50 min of incubation, 15 min before the end of the latent period, the suspension was diluted and plated for plaque formation.

UV-irradiated DNA preparations used for enhancement of transfection were added to cells after 80 min of incubation in competency medium; transfecting DNA was added 10 to 20 min later.

All competent cultures of *B. subtilis* infected with helper phages or exposed to DNA preparations were rocked gently on a specially constructed oscillating shaker. Phage adsorption to competent *B. subtilis* cells was at least 90% within 5 min.

RESULTS

As a result of his experiments with helper phages, Green (7) postulated two different enhancement mechanisms depending on whether the phages were added before or after the transfecting DNA. Phages added after the transfecting DNA were shown to require a cooperation with the DNA. This transfection enhancement was termed superinfection marker rescue. Phages added before the DNA (preinfection enhancement) were thought to act by directly inhibiting the intracellular inactivation process. This inactivation would otherwise have led to a multipowered dependence of plaque formation on the concentration of transfecting DNA.

The indirectness of Green's evidence concerning the preinfection enhancement mechanism led us to investigate the nature of the participation of the helper phages. The use of plaque-type mutant helper phages inactivated by UV irradiation made it easy to determine if the helper phage markers were rescued in the process of transfection enhancement. Two different plaque-type mutants were used: one clear and the other turbid. In contrast, the wild-type phage formed a clear-centered plaque with turbid and clear halos.

Helper phages were irradiated to approximately 10^{-7} survivors and were added to competent cells at multiplicites of up to 10 for 5 min before the addition of transfecting DNA. The results are shown in Fig. 1. Included in this figure are points for the number of added surviving helper phages forming plaques in single infection and also the number of plaques produced by the helper phages alone at the *maximal* helper-phage multiplicity. The fourfold increase in plaques formed in the absence of transfecting DNA is presumably due to multiplicity reactivation and represents only a small number compared with the number of plaques resulting from transfection enhancement. As shown in Fig. 1, a 20-fold increase in transfectants per ml occurred with a helper phage multiplicity of 8. The results always ranged between second- and third-power dependence of transfection on DNA concentration, when no helper phages were added. A fourth-power dependence was not observed.

Competent cells of *B. subtilis* infected with clear plaque-forming helper phages and wild-type transfecting DNA yielded plaques of which approximately half were mottled. To characterize the mottling, the infected cells were plated so that

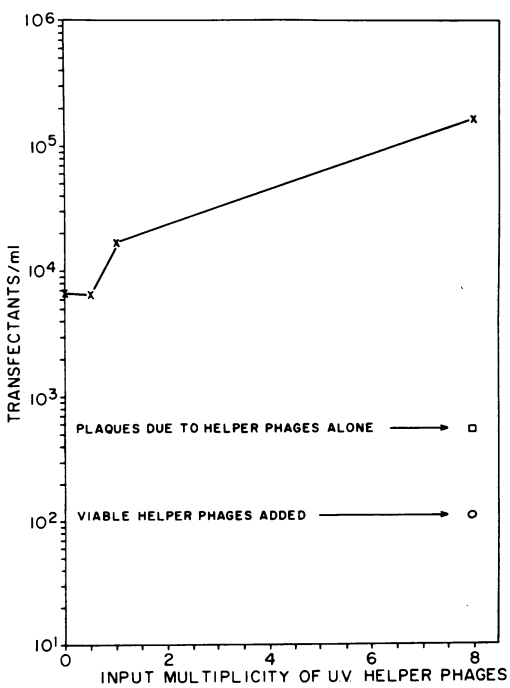

FIG. 1. *A 0.2-ml amount of UV-irradiated (10^{-7} survival) helper phages was added to incubation tubes containing 0.5 ml of competent B. subtilis cells. Ten min later, 0.1 ml of 10 μg/ml of SP82 DNA was added, and the suspension was assayed after 50 min of incubation. At the highest helper phage concentration, about 100 surviving phages were added (○) and 550 plaques were found in a control tube containing no transfection DNA (□); the increase was presumably due to multiplicity reactivation.*

40 to 50 plaques were formed on each petri plate. Picking and replating the mottled plaques revealed significant fractions (5 to 50%) of clear plaque-forming phages, indicating either recombination between the helper phages and the transfecting DNA or a marker rescue by the transfecting DNA. A similar result was found in experiments with turbid plaque-forming helper phages.

Green (6–8) presented evidence that an intracellular process progressively inactivates SP82 transfecting DNA. A similar inactivation was inferred from the finding (4) that UV irradiation of competent cells of *B. subtilis* results in a marked enhancement of transfection (5). The enhancement was interpreted as a trapping of host nucleases by the irradiated host DNA resulting in decreased attack on the incoming transfecting DNA. It seemed possible to test this mechanism by exposure of competent cells to UV-irradiated bacterial DNA before the addition of transfecting DNA.

Figure 2 shows the results of exposing competent cells to transfecting DNA, both with and without previous exposure to 1.5 μg of bacterial DNA irradiated with 2×10^4 ergs/mm² of UV light. The addition of the irradiated DNA enhanced the final yield of transfectants by a factor of 10, at the usual transfecting DNA concentration of 1 μg per 0.5 ml. As is true for the two other enhancement methods, preexposure to UV-irradiated bacterial DNA leads to a transfection which is more nearly linearly related to infectious phage DNA concentration.

The effect of increasing the concentration of irradiated bacterial DNA was maximal at about 1.5 μg of DNA per 0.5 ml of cells; this concentration was therefore adopted for all other enhancement experiments described.

The dependence of this new enhancement phenomenon on several factors was examined. Figure 3 shows the effect of varying the dose of UV administered to the 1.5 μg of bacterial DNA. Transfection enhancement reached saturation when *B. subtilis* DNA was irradiated for 5 min (2×10^4 ergs/mm²).

Figure 4 shows the results of varying the time interval between the addition of 1.5 μg of irradiated *B. subtilis* DNA and the addition of 1 μg of transfecting DNA. The time interval for maximal effect was about 20 min.

For comparison, the kinetics of transfection enhancement by the method of irradiation of competent host cells was determined. A 1-μg amount of transfecting DNA was given in 15-min pulses

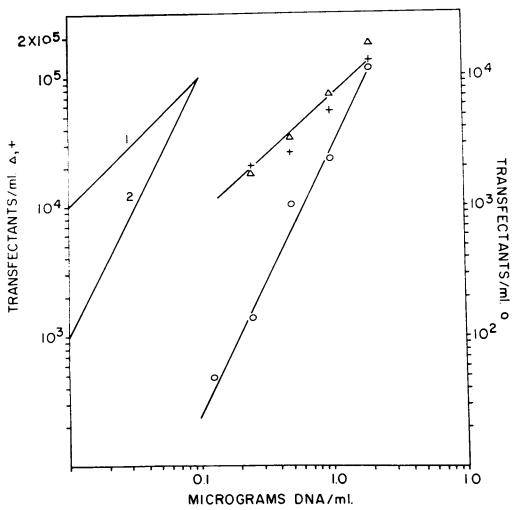

FIG. 2. *Relation between plaque-forming units and transfecting DNA concentration in 8 × 10⁸ competent cells preexposed (+) and not preexposed (○) to 1.5 μg of UV-irradiated B. subtilis DNA 10 min before addition of phage DNA. The triangles refer to an experiment with E. coli DNA to be discussed in connection with Table 1. Lines labeled 1 and 2 are theoretical first- and second-power relationships to DNA concentration. UV dose was 2 × 10⁴ ergs/mm².*

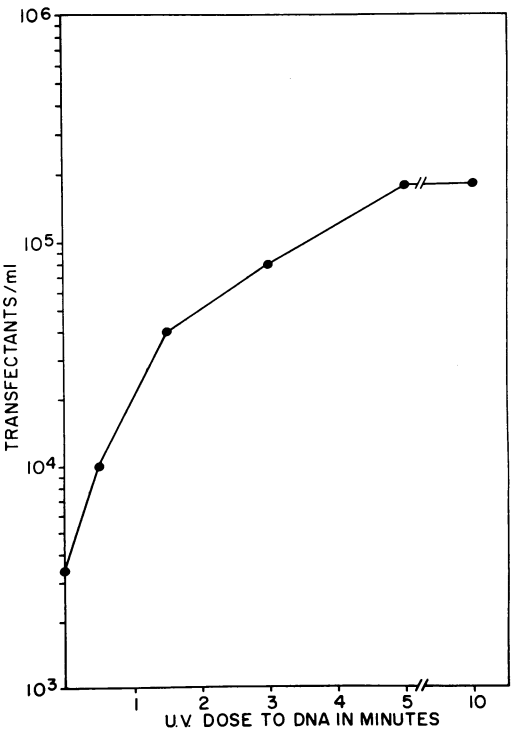

FIG. 3. *Effect of varying the UV dose given to 1.5 μg of B. subtilis DNA before adding it to 8 × 10⁸ competent cells for transfection enhancement. Dose rate was 70 ergs/mm² per sec.*

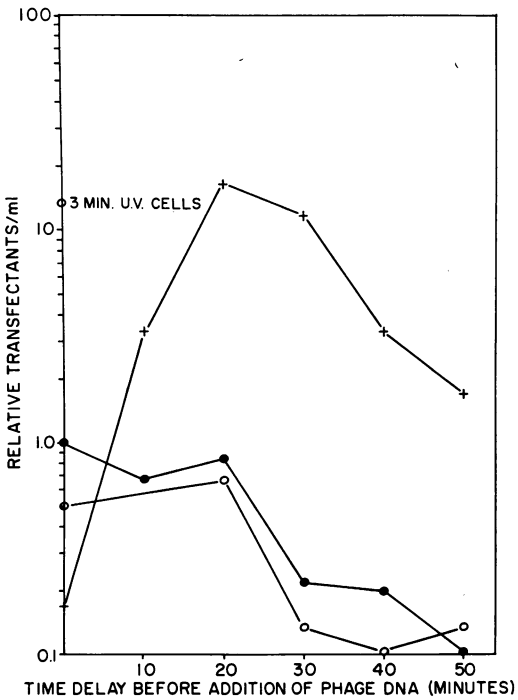

FIG. 4. *Effect of varying the time interval between the addition of 1.5 μg of irradiated [2 × 10⁴ ergs/mm² (+)] and nonirradiated (○) B. subtilis DNA and the addition of 1 μg of transfecting SP82 DNA to 8 × 10⁸ competent cells. Data (●) for adding SP82 at the various times without any preceding bacterial DNA are shown. Also shown is plaque-formation for a competent-cell sample given a 10³ ergs/mm² UV dose before addition of the transfecting DNA.*

and DNA action was terminated by the addition of 1 μg of deoxyribonuclease. The results (Fig. 5) show a similar time interval of 20 min to yield maximal enhancement.

Assuming that transfection enhancement is associated with the binding of host nucleases by the irradiated bacterial DNA, the effect should be directly correlated with the amount of photoproduct existing in the DNA. It was, therefore, of interest to measure the effectiveness of various DNA preparations which had received the same UV dose.

To study this point, UV-irradiated DNA preparations from *Micrococcus lysodeikticus*, *Escherichia coli*, *B. subtilis*, and *D. discoideum* were used. Their adenine plus thymidine (AT) contents are, respectively: 28, 50, 58, and 78% (11). The results of these experiments are presented in Table 1.

The main finding was that heterologous DNA preparations are effective in enhancing transfection in *B. subtilis*. In addition, the order of effectiveness is related to the order of AT content.

The linearization of the relation between plaque formation and SP82 DNA concentration was used as a test of similarity of action of heterologous and homologous DNA preparations. Figure 2 showed that the addition of irridated *B. subtilis* DNA produced a linear relationship. The triangles (Fig. 2) represent the data from an experiment in which irradiated *E. coli* DNA was used to enhance transfection; the result is identical to that obtained with *B. subtilis* DNA.

UV-irradiated DNA from phages SP82 and SP02 was also tested. Transfection enhancement was observed when the concentration of irradiated SP82 DNA was reduced to a level equaling phage multiplicities of 8 or less. At the usual concentration of 1.5 μg of irradiated DNA (equaling a phage multiplicity of 32), the transfection process was inhibited. The inhibition might be due to competition for replicative sites or to genetic recombination between the irradiated DNA and the transfecting DNA which could result in in

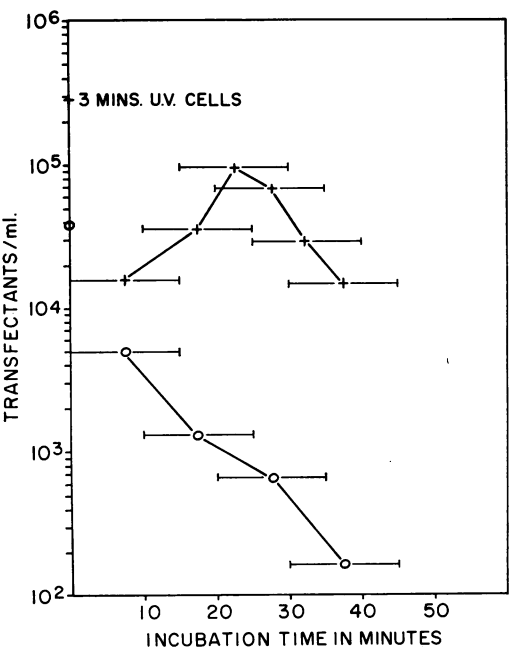

FIG. 5. *Effect (+) of adding 1 μg of SP82 transfecting DNA for 15-min periods at various times during incubation of 8 × 10⁸/ml competent B. subtilis cells given a 3-min UV dose. DNA action was terminated by the addition of 1 μg of deoxyribonuclease. Data are also given (○) for similar DNA pulses in nonirradiated cells from the same starting culture. Also shown on the ordinate are the results of leaving the DNA in contact with the nonirradiated (○) and 3-min UV-irradiated (+) cells for the entire 50-min incubation period. The UV dose corresponds to about 10³ ergs/mm².*

TABLE 1. *Effect of adding cell DNA preparations irradiated for 5 min in enhancing transfection*

Cell DNA (3 μg/ml)	Transfectants per ml	Relative transfectants
None	5×10^3	0.02
Micrococcus lysodeikticus	3×10^4	0.1
Escherichia coli	2.5×10^5	0.8
Bacillus subtilis	3.0×10^5	1.0
Dictyostelium discoideum	4.0×10^5	1.3

active products. No such competition or recombination is to be expected for SP02 DNA; the phages are unrelated and, judging by the criterion of buoyant density (10), SP02 DNA is homologous in composition to host DNA, whereas, in SP82, DNA hydroxymethyluracil replaces thymine (Kahan and Kaxan, quoted in reference 6). Therefore, irradiated SP02 DNA could be expected to act like any other photoproduct-containing DNA; in fact, it enhances transfection as well as irradiated *B. subtilis* DNA.

In all cases, addition of unirradiated DNA produced either no effect or some inhibition of transfection as shown for *B. subtilis* DNA in Fig. 4.

DISCUSSION

Enhancement of SP82 transfection can now be achieved by three methods: (i) the use of helper phages; (ii) UV irradiation of competent cells before the addition of SP82 DNA; and (iii) pretransfection exposure of competent cells to UV-irradiated DNA. All three enhancement mechanisms result in a linear dependence of plaque formation on DNA concentration.

It is possible that the latter two methods act by a similar mechanism: inactivation or binding of a host nuclease which would otherwise attack the transfecting DNA. These methods appear identical, even in the time needed for maximal expression of enhancement. Since almost 20 min are required for the peak effect, the products of the primary reaction may be involved in the enhancement.

The enhancing effect of homologous and heterologous DNA preparations irradiated with a given UV dose increased with increasing AT content of the DNA. Since it is possible that photoproducts are implicated in this effect, the amount of dimer formation in enhancing *B. subtilis* DNA was estimated.

Setlow and Carrier (12) indicate that 2×10^4 ergs/mm^2 yield approximately 5×10^4 pyrimidine dimers per *E. coli* genome. Since net dimer formation is more likely (12) with adjacent thymines than with either cytosine pairs or cytosine-thymine pairs, pyrimidine dimer formation should increase with the AT fraction of the DNA. Thus, *B. subtilis* DNA should contain more dimers than *E. coli* DNA for the same UV dose, since the relative AT percentages are 58 and 50%. This conclusion assumes, as is actually the case (2, 3), that the two DNA molecules have similar sizes. Both have molecular weights of about 2×10^9 daltons and contain about 3×10^6 nucleotide pairs. Since the AT fractions comprise about 50%, we may assume that thymidine makes up about 25% of the bases. By chance alone, 75% of the thymines will be followed by some other base. Therefore, the probability of a thymine run is $(1/4)^2 = 1/16$. Thus, there should be approximately $3 \times 10^6/16 = 2 \times 10^5$ pairs of adjacent thymines. From the dimer production data given above, *B. subtilis* DNA would contain more than 5×10^4 pyrimidine dimers after a dose of 2×10^4 ergs/mm^2. This number of dimers approaches the maximal number possible, and the UV dose could be expected to be close to the saturating dose.

It is not clear how the helper phage enhancement is related to the other two methods. Infection of competent cells of *B. subtilis* by intact phages results in plaque formation which is linear with phage concentration. The phage, therefore, must normally be able to overcome the effect which inactivates transfecting DNA. Phage-injected DNA may be functionally different or protected against inactivation.

If helper phages enhance transfection by inhibiting the intracellular inactivation process (7), such an inhibition would have to result, in our studies, from helper phages inactivated by irradiation; it is doubtful whether a functional mechanism is operating unless the phages are repaired. The time required for maximal effect of helper phages is about 14 min when computed as in the present paper. Normal phages would be presumed to act much more rapidly. Therefore, it is unlikely that the helper phages are inhibiting intracellular inactivation in the same way.

In addition to producing similar levels of enhancement, all three methods appear similar with regard to time of development of peak effectiveness. The mechanism of helper phage enhancement may thus be the same as that of the other two methods.

In preinfection experiments with UV-inactivated mutant helper phages, plaques were produced of which approximately half contained the genetic marker of the helper phage. This could result either from a marker rescue of the helper phage DNA or by recombination between the helper and the transfecting DNA. If the mecha-

nism of this enhancement is that of genetic recombination, it is possible that recombination alone might be responsible since events not resulting in rescue of the helper phage marker would remain undetected.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Science Foundation (GB4497) and the U.S. Atomic Energy Commission (contract AT30-1-3449).

LITERATURE CITED

1. Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. Cairns, J. 1963. The bacterial chromosome and its manner of replication as seen by autoradiography. J. Mol. Biol. **6**:208–213.
3. Dennis, E. S., and R. G. Wake. 1966. Autoradiography of the *Bacillus subtilis* chromosome. J. Mol. Biol. **15**:435–439.
4. Epstein, H. T. 1967. Transfection enhancement by ultraviolet light. Biochem. Biophys. Res. Commun. **27**:258–262.
5. Epstein, H. T., and L. Silver. 1968. An estimate of the action spectrum for ultraviolet enhancement of transfection. Virology **34**:382–385.
6. Green, D. M. 1964. Infectivity of DNA isolated from *Bacillus subtilis* bacteriophage, SP82. J. Mol. Biol. **10**:438–451.
7. Green, D. M. 1966. Intracellular inactivation of infective SP82 bacteriophage DNA. J. Mol. Biol. **22**:1–14.
8. Green, D. M. 1966. Physical and genetic characterization of sheared infective SP82 bacteriophage DNA. J. Mol. Biol. **22**:15–22.
9. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
10. Okubo, S., and W. R. Romig. 1965. Comparison of ultraviolet sensitivity of *Bacillus subtilis* bacteriophage SP02 and its infectious DNA. J. Mol. Biol. **14**:130–142.
11. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. **4**:430–443.
12. Setlow, R. B., and W. L. Carrier. 1964. The disappearance of thymine dimers from DNA: an error-correcting mechanism. Proc. Natl. Acad. Sci. U.S. **51**:226–231.