*EFFECTS OF DNA REPAIR IN TRANSFORMATION-HETEROZYGOTES OF PNEUMOCOCCUS**

By F. Guerrini† and M. S. Fox

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

Communicated by S. E. Luria, January 24, 1968

In an earlier report,[1] we described an analysis of the segregation products of pneumococcal transformants, whose results support the proposal that the product of transformation is a heterozygous heteroduplex structure. The present investigation confirms the proposed structure by means of an analysis not affected by nuclear multiplicity.

Let us assume that regions of bacterial DNA containing damages induced either by ultraviolet light (UV) or mitomycin C (MC) are excised and that repair synthesis makes use of the complementary strand as a template.[2-4] If a newly transformed region existed as a heterozygous heteroduplex structure, one might expect that damage in this region would, when repaired, result in the elimination of the heterozygosity to yield either homozygous transformants or recipient-type products with equal probability. The loss of transformants by conversion back to the recipient genotype would be superimposed on the lethal effects of these agents on the population and would be demonstrable if the probability of producing a lethal event were sufficiently small compared to that of introducing a repairable damage in the appropriate region. In the case of two or more linked markers the sensitivity would be even greater than that for a single marker, provided that the region of DNA excised was not long enough to result in the coincident excision of more than one marker. The effect would be expected to disappear following the first replication of the transformed population.

A more direct consequence of our proposal is that, besides a higher sensitivity of transformants to agents which damage DNA, an increasing fraction of the surviving transformants should yield pure clones, as a consequence of conversion to homozygosity.

If transformable bacteria were exposed to such damaging agents before exposure to transforming DNA, no damage should be present in the donor strand of any transformed region and tranformants should be lost only as a consequence of the loss of viability in the over-all population. Examination of the composition of clones that contain transformants would again be expected to reveal an increasing fraction of pure clones. In the case of transformants for a pair of linked markers we would expect, in addition to clones pure for both markers, clones pure for only one of them. These mixed clones would arise as the consequence of the repair of a damage affecting only one of the recipient alleles. Such clones are rarely, if ever, a normal product of transformation. The results of the experiments to be described demonstrate that all of these expectations are realized.

Methods.—The strains and media employed, the method of isolation of DNA, and the transformation procedures were those described previously.[1] The MC obtained from Calbiochem was stored at 4°C in an aqueous solution of 200 μg/ml. Exposures to the drug were in CH medium[5] at 30°C and a concentration of 0.5 μg/ml. The UV irradiation

of bacteria was also carried out in CH medium with a G.E. germicidal lamp at a dose rate of about 6 ergs/mm²/sec.

The scoring of transformants was carried out on semisolid B medium[5] in the following way: *d* and *ad* transformants were scored on plates with 80 μg/ml of sulfanilamide (SA); *ad* transformants on plates with 300 μg/ml of SA; *Sad* transformants were counted on plates with 300 μg/ml of SA, which, after 3 or 4 hr of incubation at 37°C, were overlaid with an equal volume of semisolid B medium containing 300 μg/ml streptomycin (SM) and 300 μg/ml SA; ad^+ transformants were scored on plates with 10 μg/ml of *p*-nitrobenzoic acid (NOB) and 4 μg/ml of *p*-aminosalicylic acid (PAS); ad^+ and a^+d^+ transformants, on plates with 20 μg/ml of NOB.

The collection of colonies and their genetic analyses were carried out in the manner described earlier,[1] except for the experiment described in Figures 4 and 5 in which the collection plates contained 10 ml of double-strength B medium, 5 ml of melted 3% agar, 5 ml of 0.1 *M* phosphate buffer pH 7.1 and supplemented with 30 μg/ml of SA and 1.5 μg/ml of PAS.

To test for possible MC mutagenesis, an a^+d strain was exposed to a^+d DNA, treated with MC for 1, 2, 4, or 8 min at 30°C, diluted, and incubated for 2 hr in B medium at 37°C. Among the survivors of an initial population containing 10^5 colony-forming units, no d^+ bacteria were detected at any dose. If MC exposure is mutagenic at all, it produces mutations with a frequency low enough to be irrelevant in the present context.

Results.—Sensitivity of transformants to mitomycin C: Wild-type bacteria, immediately following transformation with DNA carrying the three linked markers *S*, *a*, and *d*, were exposed for various times to MC and then diluted at least 500-fold and immobilized in semisolid medium. The small residual amount of MC is not sufficient to affect either the generation time of growing bacteria or their plating efficiency on semisolid medium.

The observations described in Figure 1 are consistent with the hypothesis of an enhanced sensitivity of transformants to MC. The survival curve of the over-all population exhibits an initial shoulder, probably due to the presence of chains in the population, then falls exponentially. The back extrapolation of the linear portion of the curve yields a calculated chain length which is in agreement with the microscopic observation of 2.4 bacteria per colony-forming unit. Bacteria transformed with respect to a single marker are initially inactivated somewhat more rapidly than are members of the over-all population. Even more rapid rates of inactivation are observed for bacteria transformed for two or three linked markers, as expected if an excision event is rarely sufficiently extensive to include more than one of the linked markers. The kinetics of inactivation of DNA carrying the three markers used here either by heat,[6] or by UV irradiation,[7] or by P^{32} disintegration[8] similarly suggests that inactivating events in any one of the markers have no effect on the survival of the others.

Any interpretation of the survival curves involving population heterogeneity so that competent cells have a specially high sensitivity to the lethal agent is excluded by the observation of the different sensitivities of the three transformant classes.

In other experiments, each of the markers examined singly manifested the same relative rate of inactivation, thus excluding interpretations involving marker differences. Furthermore, possible phenotypic peculiarities involving the method of selecting transformants were excluded by an experiment in which the donor DNA carried the markers ad^+ and the recipient bacteria were a^+d. In this case

the single-marker transformant classes examined were a^+d^+ and *ad*, and the double transformant class was ad^+. Each class manifested the MC sensitivity representative of the appropriate class in the experiment described in Figure 1.

In a manner consistent with our hypothesis, transformants that had been permitted to grow for one hour (1–1.5 doublings) were inactivated by MC at the same rate expected from the loss of viability of the over-all population.

Inactivation by exposure to UV either immediately after transformation or following growth of the transformed population yields results qualitatively similar to those observed with MC.

Transformation of cells damaged by MC: If the selective elimination of transformants by MC is indeed the consequence of events involving a repair of DNA damage, exposure of competent bacteria to MC prior to transformation should result in heterozygous structures with damages on the recipient strand only. Such structures would be expected to be converted to homozygous transformants by repair. Since no damages should be present on the donor DNA, the loss of transformants in such pretreated populations should simply reflect the loss of viability in the over-all population.

The experiment described in Figure 2 shows that this is indeed the case. A culture of competent bacteria was exposed to MC and at different times samples were filtered, resuspended in fresh medium, and transformed with a fixed concentration of DNA carrying the markers *Sad*. The over-all survival, the recovery from the filters, and the survival of the transformant classes *ad* and *Sad* were determined. Neither of the transformant classes was inactivated more rapidly than the bacteria in the over-all population. This observation excludes the possibility that the increased sensitivity of transformants to MC observed in the

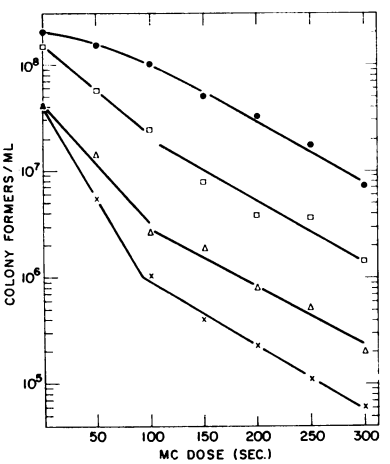


FIG. 1.—MC inactivation of a newly transformed population. Genotypes of colony formers: ● recipient; □ *d*, transformants (× 20); △, *ad* transformants (× 20); ×, *Sad* transformants (× 100).

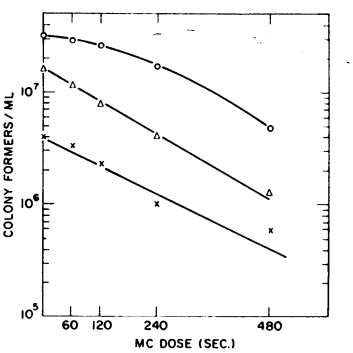


FIG. 2.—Survival of a population treated with MC prior to transformation. Genotypes of colony formers: O, recipient; △, *ad* transformants (× 40); ×, *Sad* transformants (× 400). Microscopic observation indicated 2.7 bacteria per chain.

experiments of Figure 1 is the consequence of some general effect of MC, such as the induction of an enzyme that eliminates heterozygous regions.

Analysis of the segregation products of MC-treated bacteria transformed for a single marker: In a manner similar to that described above, MC-treated competent d^+ bacteria were exposed to DNA carrying the marker *d*. The transformed culture was then subjected to ultrasound and plated in the presence of a low concentration of sulfanilamide in a manner[1] that permits the characterization of the segregation products of single viable bacteria. For each exposure to MC, 48 colonies containing *d* transformants were collected and analyzed for the presence of *d* and d^+ bacteria. As shown in Figure 3, increasing exposure of

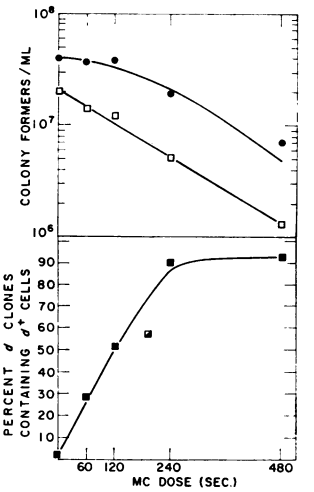


FIG. 3.—Genetic composition of clones from bacteria treated with various doses of MC and then transformed for a single marker. Genotypes of colony formers: ●, recipient; □, *d* transformants (× 10); ■, per cent of *d* clones containing only *d* cells; ◪, per cent of clones containing only *d* cells from an independent experiment involving 240 sec of MC treatment, and plotted by normalizing for lethal dose (survival 0.28). The number of clones analyzed was: 48, 48, 48, 42, 43, and 40 for the times 0, 60, 120, 240, and 480 sec, respectively. Microscopic observation indicated an average of 2.9 bacteria per chain.

recipient bacteria to MC resulted in an increasing portion of transformant clones that were pure. The frequency was half maximum for a dose that inactivated about half of the over-all population and saturated at about 90 per cent. This observation is again consistent with the expectation that pretreatment of the recipient bacteria with MC should result in the conversion of heterozygous transformants to homozygosity for the donor marker.

Analysis of segregation products of MC-treated bacteria transformed for two linked markers: Transformation of MC-treated bacteria yields transformant clones many of which are pure. In addition, the rate of MC inactivation of bacteria transformed for a pair of linked markers is consistent with the proposal that a single repair event rarely spans the distance between the two markers. From these two observations it follows that transformation of MC-treated bacteria with DNA carrying the markers *ad* should yield partial heterozygotes among *ad* transformants whenever only one of the two alleles in the recipient bacterium was damaged and then repaired. After segregation, such a transformant should yield clones containing bacteria transformed for the two linked donor markers as well as bacteria transformed for only one of them. From previous observations[1] we know that, for the *ad* linkage group, such partially heterozygous transformants occur rarely, if ever, as normal products.

In the presence of the appropriate concentrations of SA and PAS the a^+d^+ and the a^+d cells are completely inhibited, the ad^+ cells form small colonies, and the *ad* cells form colonies of normal size. These conditions permit the detection of all the *ad*-containing transformant clones in an a^+d^+ population transformed with DNA carrying the *ad* markers. It also allows the subsequent detection of those that contain some ad^+ cells since ad^+ transformants are only partially inhibited under these plating conditions.

A reconstruction experiment for detection of mixed clones containing *ad* as well as ad^+ cells was carried out by transforming chains of ad^+ cells with a^+d DNA. Large colonies from collection plates containing PAS and SA were analyzed for their genetic composition; all 19 out of 19 colonies tested contained *ad* as well as ad^+ cells.

A culture of competent a^+d^+ bacteria was then exposed to MC; at different times samples were washed by filtration, suspended in fresh medium, and transformed with a fixed amount of DNA carrying the markers *a* and *d;* the bacteria were plated for viable count, for *ad* transformants, and for detection of partially converted clones containing both *ad* and ad^+ cells. Colonies to be analyzed were collected only from plates whose SA and PAS concentrations yielded numbers of small and large colonies consistent with the numbers observed on control plates on which *ad* and ad^+ transformants were independently scored. The experimental results described in Figure 4 show that pretreatment with MC results in partial conversion of transformants that had incorporated the *ad* linkage group.

The results of a similar experiment where UV replaced MC as the damaging agent are described in Figure 5. This experiment was carried out in a manner similar to those using MC except that washing by filtration was omitted. The frequency of clones carrying both *ad* and ad^+ bacteria rose with dose from a value too small to be detected to a maximum of about 20 per cent. At larger doses, the frequency again fell to low values, as expected when the number of damaging events in the resident genome approaches saturation.

Examination of the segregation products of MC-treated transformants: The results of similar experiments involving exposure of newly formed transformants to MC also demonstrate the elimination of heterozygosity. However, in this case the fraction of transformed clones that contain pure transformants reaches a maximum of only about 50 per cent even after long exposures to MC. The experiment was carried out by transforming d^+ bacteria with DNA carrying the *d* marker and then treating them with MC. At various times samples were filtered, resuspended, and plated for viable count and for *d* transformants. The bacteria were also sonicated for eight minutes, yielding about 10^{-3} survivors, and plated for detection of mixed clones as described earlier.[1] The results are described in Figure 6.

The killing curve of the *d* transformants is similar to that described in Figure 1. Increasing exposures to MC result in more and more of the surviving transformants yielding pure clones. The frequency of transformant clones that are pure reaches a plateau value of about 0.5 and reaches its half maximum at an MC exposure that gives about 50 per cent survival. This observation is to be con-

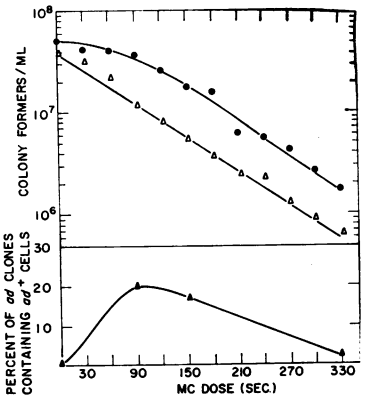


FIG. 4.—Genetic composition of clones from bacteria treated with various doses of MC and then transformed for two linked markers. Genotypes of colony formers: ●, recipient; △, *ad* transformants (× 100); ▲, per cent of *ad* clones containing *ad*+ cells. The number of clones containing *ad* transformants, subsequently analyzed for the presence of *ad*+ cells was 94, 95, 96, and 90 for the times 0, 90, 150, and 330 sec, respectively. Microscopic observation indicated 3.4 bacteria per chain.

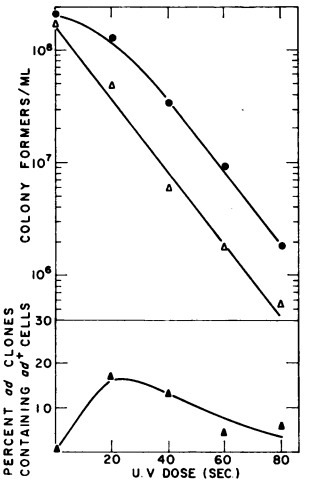
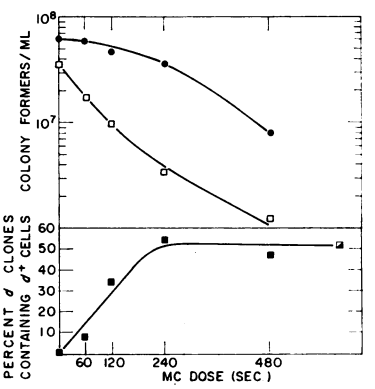


FIG. 5.—Genetic composition of clones from bacteria treated with various doses of UV and then transformed for two linked markers. Genotypes of colony formers: ●, recipient; △, *ad* transformants (× 200); ▲, per cent of *ad* transformants, subsequently analyzed for the presence of *ad*+ cells was: 96, 90, 92, 76, and 62 for the times 0, 20, 40, 60, and 80 sec, respectively. Microscopic observation indicated 3.1 bacteria per chain.

trasted with the observation that, when MC treatment precedes transformation, up to 90 per cent of the surviving transformants yield pure clones.

Discussion.—The kinetics of MC inactivation of transformants was found to be consistent with the expected consequences of damages followed by excision and repair within heterozygous heteroduplex DNA structures. Such damage and repair is expected to yield pure clones of transformants. In the case of transfor-

FIG. 6.—Genetic composition of clones from bacteria transformed and then treated with various doses of MC. Genotypes of colony formers: ●, recipient; □, *d* transformants (× 20); ■, per cent of clones containing only *d* cells; ◪, per cent of clones containing only *d* cells from an independent experiment involving 480 sec of MC treatment and plotted by normalizing for lethal dose (survival 0.012). The number of clones analyzed was: 45, 48, 48, 48, 44, and 48 for the times 0, 60, 120, 240, 480, and 480 sec (second independent experiment), respectively. Microscopic observation indicated 3.1 bacteria per chain.



mation of MC-treated bacteria, pure clones were found and their frequency reached a maximum of about 90 per cent at a dose that reduced the viability of the over-all population to about 20 per cent. The possibility that these pure clones arise as a consequence of a lethal event in the untransformed nucleus of a presumptive binucleate transformant was excluded by the results of an examination of the segregation products of bacteria transformed with respect to a pair of linked markers.

Among 76 clones arising from bacteria transformed for both members of the *ad* linkage group without damaging treatments, none had been found to contain bacteria transformed for only one of these markers.[1] In the present investigation, only two such mixed clones were observed among 190 examined. Since the plating method employed here did not require the disruption of bacterial chains, two independent transformation events could readily account for the mixed clones observed.

Following transformation of MC-treated bacteria, mixed clones containing two transformant types occurred in substantial numbers. The frequency of such clones increased as a function of the time of exposure to MC and reached a maximum for an exposure that resulted in about one lethal hit in the total population. The occurrence of such mixed clones requires that the MC-induced damage and repair occur in the region of the particular segment of DNA that participates in the transformation event. It further requires that the repair process utilize a portion of the complementary undamaged DNA strand as a template. Since the dose response for production of the mixed clones by bacteria transformed for a pair of linked markers was the same as that for the production of pure clones when a single marker was examined, it appears unlikely that these two processes are different in origin. Since 90 per cent of the heterozygotes are eliminated during the first posttransformation bacterial doubling,[1] it appears that heterozygotes replicate semiconservatively and rarely, if ever, yield heterozygous products.

The subsequent decline in the frequency of clones containing two transformant types is consistent with the notion that increasing doses result in an increased probability of a damage that results in conversion of both the recipient alleles. It is possible to estimate the maximum expected frequency of clones containing two transformant types on the assumption that the repair of MC or UV damage rarely involves a polynucleotide stretch that includes both of the linked markers used. We further assume that gross killing events occur with equal probability in all members of the bacterial population.

The probability p of finding a damage opposite the a region and not finding a damage opposite the d region in an *ad* transformant is $p = (1-e^{-m})e^{-m}$, where m is the average probability of a damage occurring in either region. The maximum expected value of p can be determined by calculating the value of m for which $dp/dm = 0$; this yields a value of 0.25. The observed maximum frequency of clones with both a and *ad* transformant types is 0.2, in reasonable agreement with the predicted value. These observations suggest that the damages by MC or UV result in excision of polynucleotide sequences whose average length is shorter than the physical distance between the markers a and d.

The maximum frequency of clones containing two transformant types occurs at

a dose delivering about one lethal hit to the over-all population. At this dose, the calculation indicates that each region heterozygous for a single marker has received an average of 0.5 repairable damages. A similar assessment results from the examination of the frequency of pure clones as a function of MC dose when a single marker is tested. This suggests that a very large number of repairable damages occur for each lethal event. Since DNA damage so frequently results in conversion to homozygosis of a portion of a heterozygous heteroduplex recombination product, events of this kind may account for the increase in recombination between closely linked markers observed following UV treatment.[9]

The frequency of pure transformant-containing clones in MC-treated bacteria reaches a maximum level of 0.9. Since it is likely that the bacterial genome has a unique origin of replication, various markers should appear more or less frequently in the replicated region of a genome. Transformation occurring on the already replicated region should never yield pure clones by conversion unless it were possible to "kill" one of the replicated arms. The remarkably high frequency of pure clones that has been observed leads us to suggest that the *d* marker occurs near the terminal point of replication and is, therefore, only rarely (10%) present in two copies in a transformed bacterium.

It might have been expected that exposure to MC following transformation would yield a similarly high frequency of pure clones among surviving transformants. Under those conditions the frequency of pure clones does rise with increasing dose and also levels off at about 1.5 lethal hits, but at a value of only about 0.50. Under these circumstances, the donor fragment of DNA is also subject to damage by the MC. The loss of capacity for conversion of about half of the transformants might be the consequence of a unique direction of excision, whose extent is limited by the existence of a damage on the opposite strand. This hypothesis would lead to a dose-dependent reduction of excision length and to saturation of the frequency of converted clones at an intermediate frequency, even at infinite MC doses.

Summary.—Further evidence has been presented supporting the heteroduplex heterozygote structure of the recombinant region of DNA in transformed bacteria. It has also been demonstrated that under normal circumstances conversion does not occur prior to replication. Following treatment with MC or UV, conversion does occur, indicating that repair following excision takes place on the template of the complementary strand.

* This investigation was supported by National Institutes of Health grant AI-05388.

† Present address: International Laboratory of Genetics and Biophysics, Naples, Italy.

[1] Guerrini, F., and M. S. Fox, these PROCEEDINGS, **59**, 429 (1968).
[2] Setlow, R. B., and W. L. Carrier, these PROCEEDINGS, **51**, 226 (1964).
[3] Pettijohn, D., and P. Hanawalt, *J. Mol. Biol.*, **9**, 395 (1964).
[4] Boyce, R. P., and P. Howard-Flanders, *Z. Vererbungslehre*, **95**, 345 (1964).
[5] Gurney, T., and M. S. Fox, *J. Mol. Biol.*, in press.
[6] Kent, J. L., M. Roger, and R. D. Hotchkiss, these PROCEEDINGS, **50**, 717 (1963).
[7] Fox, M. S., unpublished results.
[8] Fox, M. S., *J. Mol. Biol.*, **6**, 85 (1963).
[9] Jacob, F., and E. Wollmann, *Ann. Inst. Pasteur*, **88**, 724 (1955).