Radiation-sensitive and Radiation-resistant Mutants of Haemophilus influenzae

B. J. BARNHART AND S. H. COX

Biomedical Research Group, Los Alamos Scientific Laboratory, University of California,
Los Alamos, New Mexico 87544

Received for publication 29 April 1968

Most of the recent investigations on the genetic control of radiation sensitivity have been done with bacteria and related bacteriophages. More specifically, the majority of these studies have included various strains of Escherichia coli (H. I. Adler, Advan. Radiation Biol. 2:167, 1966). Comparable information on other microorganisms is limited. This note relates the isolation and description of an ultraviolet (UV)-sensitive mutant and an UV-resistant mutant of Haemophilus influenzae strain Rd (H. E. Alexander and G. Leidy, J. Exptl. Med. 97:17, 1953).

The resistant mutant was isolated from a logarithmic-phase culture irradiated with a dose of UV from a GE germicidal lamp (model G-30T8), permitting 0.002% survival. From the survivors, 20 colonies were inoculated into 3% Difco Brain Heart Infusion medium, supplemented with 10 μg of hemin per ml and 2 μg of nicotinamide adenine dinucleotide per ml (BH medium), and grown overnight. This incubation and all other incubations were at 37 C, and liquid broth cultures were routinely shaken on a reciprocal shaker. The overnight cultures were diluted 1:50 into fresh BH medium, grown to the logarithmic phase, resuspended in saline, and UV-irradiated with a dose permitting 0.6% survival of the wild-type strain Rd. Two cultures showed survival of over 3%. Upon examination, only one of these proved to be more UV-resistant than the wild type.

The sensitive mutant was isolated as follows. Strain Rd was grown in BH medium to the logarithmic phase; at that time, 10 μg of N-methyl-N'-nitro-N-nitrosooguanidine (NG) per ml was added. After 10 min of incubation, the culture was diluted and spread on the surface of BH agar. After overnight incubation, 532 colonies appeared; these colonies represented 0.5% survival of the NG-treated culture. They were replicated onto BH agar plates, immediately exposed to an additional mutagen, UV irradiation, and then incubated. From the survivors, 18 colonies were suspended in BH medium and grown overnight. To assay for UV sensitivity, the overnight cultures were diluted 1:50 into fresh BH medium and were grown to the logarithmic phase; they were resuspended in saline, and 5.0-ml quantities were irradiated with a dose of UV permitting 3.4% survival of the wild-type strain Rd. Fifteen of the cultures showed sensitivity similar to Rd; two were 1.5 and 2 times more resistant but later were determined to be Rd; and one resulted in 0.005% survival. This mutant, which is designated BC100, and the resistant mutant, BC200, were subcultured five times in liquid medium; each subculture was inoculated from a colony isolate of the previous culture. There were no variations in the UV response of these strains, indicating that they are genetically stable. The mutant strains currently and hereafter designated BC100 and BC200 were partially described and designated Rd, and Rd/r, respectively, in a previous communication (B. J. Barnhart and S. H. Cox, Bacteriol. Proc., p. 36, 1968).

UV dose-survival curves, giving a comparison of strains Rd, BC100, and BC200, are shown in Fig. 1. The rates of inactivation of strains Rd and BC100 decreased at relatively higher doses. The breaks in the curves occurred at 45 ergs/mm² and a survival of 10^{-6} for strain BC100 and at 240 ergs/mm² and a survival of 6 × 10^{-3} for strain Rd. The slope of the slower component of the Rd inactivation curve was somewhat increased when 10⁴ viable centers/ml were irradiated, as opposed to 10⁰ or 10⁴ per ml used in these experiments; however, the multicomponent nature of the curve and the relative sensitivities of the three strains were unchanged. Irradiation of cultures grown from colony isolates of survivors, represented by the slower components of Rd and BC100 curves, showed the same sensitivities as the parental cultures. Thus, these multicomponent curves describe the inactivation of cell populations which are genetically homogeneous with respect to UV sensitivity. The inactivation rate of BC200 describes a single exponential down to 10^{-6} survival.
The UV source was an unfiltered GE 30-w germicidal lamp (model G-30T8) emitting mainly at 254 nm. The incident dose rate was calculated to be 30 ergs/mm² per sec, as determined by readings from a Keithley model 150B microvolt-ammeter connected to an Eppley thermopile calibrated by the National Bureau of Standards. For irradiation, logarithmic-phase cultures (optical density at 650 nm Rd (wild-type), strains G-30T8 (UV-sensitive), and BC200 (UV-resistant). The UV source was an unfiltered germicidal lamp (model G-30T8) emitting mainly at 254 nm. The incident dose rate was calculated to be 30 ergs/mm² per sec, as determined by readings from a Keithley model 150B microvolt-ammeter connected to an Eppley thermopile calibrated by the National Bureau of Standards. For irradiation, logarithmic-phase cultures (optical density at 650 nm = 0.4) were centrifuged and resuspended in 2 volumes of saline, giving approximately 10⁹ viable centers/ml. Suspensions were irradiated in 9-cm glass petri dish bottoms in volumes of 5 ml (1 mm depth) and were continuously agitated in a reciprocal motion. Fraction surviving: that fraction of cells retaining the ability to form colonies when plated on BH agar.

X-ray dose-survival experiments showed that Rd and BC200 had almost equal sensitivities. BC100 was more sensitive but did not demonstrate the extreme sensitivity that was observed for UV inactivation.

The plating efficiency of Haemophilus phage HP1cl (originally obtained from C. S. Rupert) was similar on the three host strains. However, the UV doses required to reduce phage survival to 0.37 on BC100, Rd, and BC200 were 105, 240, and 240 ergs/mm², respectively. The phage inactivation curves displayed two components for plaque-forming ability on all three strains.

It has been reported that strain Rd is able to reanimate UV-irradiated deoxyribonucleic acid (DNA) of bacteriophage (W. Harm and C. S. Rupert, Z. Vererbungslehre 94:336, 1963), transforming DNA, and the DNA of its own chromosome (M. H. Patrick and C. S. Rupert, Photochem. Photobiol. 6:1, 1967). The isolation of UV-sensitive and UV-resistant mutants permits a more definitive demonstration of repair of UV damage. Figure 2 shows that the major difference in sensitivity of Rd and BC200 is due to varying capacity for repair of UV damage to the bacterial DNA. The same fraction of Rd and BC200 cells form colonies if plated immediately after irradiation in agar containing acriflavine, a known inhibitor of repair in E. coli (R. R. Feiner and R. F. Hill, Nature 200:291, 1963). However, the fraction surviving is much greater if the cells are incubated in nongrowth liquid (liquid-holding) between UV irradiation and plating, especially in strain BC200 when plated in the presence of acriflavine.
The recovery of colony-forming ability approaches the same level whether or not the cells are allowed to undergo additional repair after plating. The survival of the sensitive strain BC100 increased steadily from $10^{-2}$ to $3 \times 10^{-2}$ over a 240-min liquid-holding period. Thus, while liquid-holding allows recovery, it appears that repair or recovery of DNA synthesis, or both, proceed at a greatly reduced rate in BC100 relative to the wild-type. Plating in acriflavine-agar after liquid-holding resulted in an initial ninefold reduction and a final threefold reduction in survival of the sensitive strain.

After completion of this investigation, the following communications reporting UV-sensitive mutants of *Haemophilus* appeared in the literature: J. K. Setlow et al., and M. E. Boling et al., Biophys. Soc. Abstr. 12:54, 1968; J. K. Setlow et al., J. Bacteriol. 95:546, 1968; R. S. Day III and C. S. Rupert, Biophys. Soc. Abstr. 12:51, 1968. The UV-sensitive mutants reported by these investigators and the UV-sensitive and UV-resistant mutants presented in this paper bring the number of *Haemophilus* mutants displaying variable UV response to at least 10. These mutants should be extremely helpful in studies of genetically controlled repair of UV damage. A detailed investigation of UV dark-repair in *H. influenzae*, employing genetic transformation and the newly developed strains reported here, is in progress.

This investigation was performed under the auspices of the U.S. Atomic Energy Commission.