Further Studies of Ultraviolet-sensitive Mutants of *Escherichia coli* Strain B

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SUMMARY

A group of twelve ultraviolet-sensitive mutants has been isolated after u.v. irradiation of *Escherichia coli* strain B. The use of crystal violet during irradiation and subsequent growth appears to increase the frequency of such mutants among colonies formed by surviving cells. This effect may be due to post-irradiation selection. Eleven of the mutants were more crystal violet resistant than their parent.

The mutants were compared by determining their u.v. survival curves, the extent of elongation after u.v. irradiation, their ability to repair u.v. induced damage to bacteriophage T1, and their resistance to furacin. They comprised nine different phenotypes. In all but one case, the mutants differed from the parent in more than one property.

INTRODUCTION

The genetic basis of ultraviolet (u.v.) sensitivity in the bacterium *Escherichia coli* has been established in a number of recent investigations (Adler & Copeland, 1962; Rörsch, Edelman, van der Kamp & Cohen, 1962; Howard-Flanders, Boyce, Simson & Theriot, 1962; Rörsch, Edelman & Cohen, 1963). These studies have usually consisted in the genetic transfer of properties previously found in some u.v. sensitive or u.v. resistant mutants and the subsequent demonstration that the recipient organism has acquired the expected change in u.v. sensitivity. The properties employed have been the ability to repair u.v. induced damage in bacteriophage and the formation of snake-like filaments after u.v. irradiation. The former property is thought to be dependent upon the organism's ability to repair u.v. induced damage to DNA; the latter is considered to be indicative of u.v. induced damage to cell division.

It is apparent from these studies that u.v. radiation produces more than one effect. Hence it might be expected that mutants whose increased u.v. sensitivity is due to increased susceptibility to one effect should be like the parent strain with respect to susceptibility to other effects. The present report concerns an investigation of this question in the case of u.v. sensitive mutants derived from *E. coli* strain B.

METHODS

The bacterial strains employed were *E. coli* B, a u.v. resistant (B/r) mutant, and twelve u.v. sensitive (B_s) mutants. Two of the u.v. sensitive mutants, B_{s-1} and B_{s-2}, had been isolated previously and some of their properties described (Hill & Simson,

1961). The ten additional mutants were isolated as follows. Cultures of B were grown to the logarithmic stage by aeration in nutrient broth. Samples containing 10^6 – 10^7 organisms were spread on nutrient broth agar. In some isolation experiments the nutrient broth agar was supplemented with crystal violet. The significance of this will be discussed later.

After spreading, the organisms were u.v. irradiated with doses allowing 10^{-4} – 10^{-5} survival and incubated at 37° . Colonies formed by surviving cells were subcultured in nutrient broth and loopfuls were spread over small areas on nutrient broth agar. These areas were u.v. irradiated with a dose (160 ergs/mm.²) allowing 1 % survival of B cells. After 3 hr incubation, the spread areas were examined microscopically. Areas containing very short cells or mixtures of short cells and slightly elongated cells were tentatively diagnosed as containing B_s mutants, in comparison with areas showing extremely long filaments ('snakes'), which is typical of B at the given dose, or with areas showing a moderate amount of cellular elongation and microcolony formation, typical of B/r at the same dose (Payne, Hartman, Mudd & Phillips, 1956; Hill & Simson, 1961).

The tentative diagnosis of B_s mutants was confirmed or refuted by macroscopic re-examination of the spread areas, after re-incubation overnight. Areas containing B or B/r showed dense confluent growth while areas containing u.v. sensitive mutants showed considerably less growth. The most sensitive showed either no growth or only a few isolated colonies. Occasionally, areas which had been tentatively diagnosed as containing B_s mutants showed a faint film of growth. Subsequent determination of the survival curves showed that these were slow-growing B/r types. In two cases, the tentative diagnosis of B on the basis of the formation of snakes was refuted by the scanty growth overnight and the survival curves for these showed that they were B_s mutants.

The procedure used to determine the u.v. survival curves for the bacteria was the same as the preceding except that measured samples were spread on nutrient broth agar and irradiated with a range of doses. Bacteriophage T1 was irradiated while suspended in 2 % ammonium acetate. Its survival, using the different bacterial strains as hosts, was determined by the agar layer method (Adams, 1950). The apparatus used for irradiation has been described previously (Hill & Rossi, 1952). The present dose rate of the u.v. lamp at 56 cm. is 8.1 ergs/mm.²/sec. as measured by a calibrated Jagger dosimeter (Jagger, 1961).

The extent of elongation of *Escherichia coli* cells after u.v. irradiation is dependent upon the strain and the dose (Payne *et al.* 1956). In order to compare more precisely the ability of the different B_s mutants to elongate, the procedure described above for the tentative diagnosis of type was modified. After the survival curves were obtained, each mutant was irradiated with a dose giving about 1 % survival. The extent of elongation by 3 hr after irradiation was then classified as 1+ to 4+ by comparison with the length (4+) of the snakes formed by B at 1 % survival.

Sensitivity to crystal violet was determined by spreading 0.1 ml. samples of appropriate saline dilutions of broth-grown cultures on nutrient broth agar containing varying concentrations of the dye, followed by incubating at 37° . Exposure to crystal violet during growth slows the development of colonies. By counting the numbers of colonies appearing by a maximum of 4 days of incubation, survival

curves for colony formation as a function of dye concentration were obtained. The times required for the appearance of colonies were also noted.

Sensitivity to furacin was determined by the gradient plate technique (Szybalski, 1952).

RESULTS

Effect of crystal violet on the isolation of B_s mutants

The numbers of B_s, B/r and parental B colonies found in different isolation experiments are shown in Table 1. The presence of B/r colonies could be due to selection of pre-existing mutants as well as to the induction of new mutations of this type. Selection undoubtedly occurs since B/r mutants are more resistant both to u.v. radiation and to crystal violet than B (Bryson *et al.* 1951–52). Unfortunately, the evidence available on the u.v. induction of B/r mutations in B organisms is scanty and equivocal (Witkin, 1947). In two reported experiments, the frequencies of B/r mutants before and after u.v. irradiation of B were determined. In one case,

Table 1. *Frequency of radiological types among survivors of u.v. irradiation of Escherichia coli B*

Expt.	u.v. dose (sec.)	Concen- tration of crystal violet (μg./ml.)	No. of colonies examined	No. of colonies of		
				B/r	B _s	B
1	80	1	11	1	1	9
2a	80	0	100	7	1	92
b	0	0	100	0	0	100
3a	60	2	27	23	3	1
b	40	2	54	47	2	5
c	0	2	217	107	0	110
d	80	1	60	13	2	45
e	60	1	21	5	1	15
4	60	0	132	3	2	127

the post-irradiation frequency was about ten times the frequency expected on the basis of u.v. selection, as calculated from the survival curves. However, in the second, the post-irradiation frequency was very nearly equal to the expected frequency. In the absence of definite information to the contrary, it was assumed that B/r mutations may not be u.v. inducible in B and therefore that the presence of B/r mutants among surviving colonies was due only to selection. These colonies were regarded as unavoidable ‘contaminants’.

The significance of this is that in calculating the frequency of B_s colonies, B/r colonies should not be included in the total scored if they are merely contaminants. However, the possibility that some of the B_s colonies might have resulted from u.v. induced mutation in the B/r ‘contaminants’ also has to be considered. The unlikelihood of this can be demonstrated. For example, in experiments 3a and 3b (Table 1), 27 and 54 total colonies respectively were scored. In each case, these numbers were the totals appearing after the irradiation of 10⁷ organisms. The spontaneous frequency of B/r in populations of B is about 10⁻⁵ (Witkin, 1947). Thus the

total number of irradiated organisms must have included about 100 B/r mutants. Since only three and two B_s colonies were found in experiments 3a and 3b respectively, the u.v. induced mutation rate for the change from B/r to B_s would have to be extremely high. However, in separate experiments where B/r populations were irradiated to 1 % survival in the presence of 1 µg. crystal violet per ml. of nutrient broth agar, no B_s mutants were found among 412 colonies formed by surviving cells. A similar result has been reported for the u.v. irradiation of *E. coli* strain K 12, a strain resembling B/r in u.v. resistance. No u.v. sensitive mutants were found among 500 colonies (Howard-Flanders & Theriot, 1962). Thus although u.v. sensitive mutants may be u.v. induced in B/r, the rate of induction cannot be high enough to account for the B_s mutants found after the u.v. irradiation of B populations. The B_s mutants must therefore have resulted from mutation in B only.

In view of the above considerations, the frequency of B_s mutants was calculated as the ratio of B_s colonies to the total of B and B_s colonies. As shown in Table 2, the frequency is increased markedly when increasing concentrations of crystal violet are present during irradiation and subsequent growth.

Table 2. *Effect of crystal violet concentration on frequency of B_s mutants*

Concentration of crystal violet (µg./ml.)	B _s /B _s + B* (%)	Mean
0	1.0	
0	1.6	1.3
1	10.0	
1	4.3	
1	6.7	7.0
2	75	
2	30	53

* The reason for excluding B/r mutants from the denominator of this fraction is explained in the text.

Properties of B_s mutants

The u.v. survival data for small and large doses to the B_s mutants are shown in Fig. 1. Although there was a wide distribution in the positions of the curves and variation in shape, all of the mutants were more u.v. sensitive than the parent B strain (Fig. 1b).

The curves for the survival of colony formation when the strains were grown on agar containing varying concentrations of crystal violet were of three types. The strains were therefore compared by assigning each to a type, as illustrated in Fig. 2. Type A includes strains whose survival shows a sharp decrease at crystal violet concentrations of 1–2 µg./ml. Type B strains show a sharp decrease at 2–4 µg./ml. The survival curves for strains of type C show a gradual decrease for concentrations up to at least 8 µg./ml. Type C is heterogeneous; some strains showed an accelerated decrease in survival at 8–16 µg./ml. while others continued to decrease at the same rate even at concentrations as high as 32 µg./ml. In addition, colonies of the latter appeared within 24 hr even at 32 µg./ml. Thus, type C strains could be distinguished as C₁, C₂ and C₃, in order of increasing resistance.

Of the twelve mutant strains, nine were classified as type C, two as type B and only one as type A, the most crystal violet-sensitive group. The only other type A strain was the parent B (Table 3). Thus eleven of the mutants were more crystal violet-resistant than their parent. The only strain (B_{8-11}) which was like the parent,

Fig. 1 (a)

Fig. 1 (b)

Fig. 1. Survival of u.v. sensitive mutants of *Escherichia coli* B after small and large doses of u.v. radiation. Dotted curves are for those mutants which are unable to repair u.v. induced damage in bacteriophage T1 (hcr^-).

Table 3. *Properties of B_8 mutants*

Strain	Isolated in expt.*	Elongation after u.v. irradiation	Sensitivity to crystal violet (type)	Inhibitory concentration of furacin ($\mu\text{g./ml.}$)	Ability to repair T1 (hcr^+ or hcr^-)
B	—	4+	A	0.25	+
B/r	1	2+	C3	3.3	+
B_{8-1}	1	1+	C2	0.23	—
B_{8-2}	2	1+	C1	0.53	+
B_{8-3}	3a	4+	B	0.25	—
B_{8-4}	3a	2+	C2	0.75	+
B_{8-5}	3a	2+	C2	0.67	+
B_{8-6}	3b	2+	C2	0.53	+
B_{8-7}	3b	2+	C2	0.59	+
B_{8-8}	3d	2+	C3	0.87	—
B_{8-9}	3d	2+	C3	0.59	+
B_{8-10}	3e	2+	C2	0.73	+
B_{8-11}	4	1+	A	0.20	+
B_{8-12}	4	4+	B	0.20	—

* See Table 1.

had been isolated without the use of crystal violet. However, this is probably without significance since, although B_{S-2} and B_{S-12} were also isolated in this way, they were classified as types C and B, respectively.

Two different survival curves were obtained when the mutants were used as hosts for u.v. irradiated bacteriophage T1. The change in slope shown in the upper curve of Fig. 3 has been interpreted as indicating that the host organism can repair

Fig. 2

Fig. 2. Types of survival curves shown by B_s mutants when grown in the presence of crystal violet.

Fig. 3

Fig. 3. Survival of u.v. irradiated bacteriophage T1 when measured by using different B_s mutants as host organisms.

a fraction of u.v. induced damage in T1 (Garen & Zinder, 1955; Harm, 1963). Strains giving this curve have been designated as *hcr*⁺ (host cell reparability positive). The lower curve of Fig. 3 is characteristic of *hcr*⁻ strains. Eight of the mutants gave the same *hcr*⁺ curve and four gave the same *hcr*⁻ curve.

In regard to sensitivity to furacin, four of the mutants were as sensitive as the parent and eight were more resistant (Table 3). None of the latter were as resistant as the B/r strain used for comparison. Similarly, none of the mutants of type C with respect to crystal violet resistance were as resistant as this B/r. Therefore other B/r strains in our collection were examined. One B/r, a tryptophan-deficient auxotroph used in other studies, showed a crystal violet survival curve identical to that shown by B_{S-2} . Two other B/r mutants were inhibited by about 0.75 μ g. furacin/ml., as were two of the B_s mutants.

Comparison of the twelve B_s mutants (Table 3, Figs. 1 and 2) showed possibly nine different phenotypes. Four of these were represented by the four *hcr*⁻ strains. Although B_{S-3} and B_{S-12} were identical in snake formation and antibiotic resistance,

their u.v. survival curves were distinctly different. B_{8-1} and B_{8-8} differed from B_{8-3} and B_{8-12} in snake formation and crystal violet resistance and differed from each other in crystal violet resistance, in furacin resistance and in their u.v. survival curves. The eight hcr^+ strains gave an additional five phenotypes. B_{8-11} differed from all the other hcr^+ strains in that it had the same sensitivity to the antibiotics as the B parent. B_{8-9} was like the hcr^- B_{8-8} in having the greatest resistance to crystal violet and in addition, had the greatest resistance to high doses of u.v. radiation of all the B_8 strains. Of the other six hcr^+ strains, B_{8-2} could be differentiated by its crystal violet and u.v. curve and B_{8-6} by the fact that, although it was like B_{8-4} , B_{8-5} , B_{8-7} and B_{8-10} in degree of elongation and antibiotic resistance, its u.v. curve was very close to that for B_{8-2} . Only B_{8-4} , B_{8-5} , B_{8-7} and B_{8-10} may have been identical.

DISCUSSION

The finding that B_8 mutants tend to be more crystal violet resistant than B suggests that the mechanism by which crystal violet increases their frequency among colonies formed by u.v. irradiated B organisms is probably selective. This would not be a selection of pre-existing mutants. Rather, those parental organisms which survive irradiation but mutate to the B_8 form would have a selective advantage during subsequent growth in the presence of the dye over those survivors which do not mutate. Since the use of crystal violet alone does not select B_8 mutants to a detectable extent (Table 1), it seems likely that they originated by u.v. induced mutation rather than by spontaneous mutation during post-irradiation growth on agar. In this respect, mutation to increased u.v. sensitivity may differ from mutation to increased u.v. resistance since, as mentioned previously, the latter may not be u.v. inducible.

The wide distribution which was observed for the positions of the u.v. survival curves is independent of whether the B_8 mutants are hcr^+ or hcr^- . A similar spread has been reported for hcr^- u.v. sensitive mutants of *Escherichia coli* K 12 (Howard-Flanders & Theriot, 1962). It has been suggested that the hcr property is determined by the ability of the organism to produce an enzyme for the repair of u.v. induced damage to DNA (Sauerbier, 1961). The fact that hcr^- mutants are not equally u.v. sensitive may mean that the amount of this enzyme is more critical for the survival of the u.v. irradiated bacterium. In addition, it seems clear that u.v. sensitivity is determined by a number of factors rather than by only one (Howard-Flanders & Theriot, 1962). A second factor which has been proposed is injury to cell division, manifested by the formation of snake-like filaments after irradiation. According to cytological studies, 'snake' formation is due to continuous nuclear replication and cytoplasmic increase without cell division (Payne *et al.* 1956). A third factor is suggested by the present finding that the hcr^- mutants B_{8-3} and B_{8-12} formed 'snakes' after u.v. irradiation like the parent B strain. Apparently these mutants can replicate their DNA in spite of inability to repair it. It has been suggested that DNA replication without repair results in mutation (Witkin, 1961). Therefore the increased u.v. sensitivity of B_{8-3} and B_{8-12} might be due to a combination of the hcr^- property and the production of new mutations which subsequently reduce the probability of recovery from the u.v. induced damage to cell division even more than it is reduced in the case of the hcr^+ parent B.

Injury to cell division (manifested by snake-formation) is produced by exposure to other agents besides radiation. These include crystal violet and furacin. It has been suggested that snake formation and sensitivity to these antibiotics are correlated properties (Payne *et al.* 1956; Rörsch *et al.* 1962). However, the present results show that this is not always the case. Although B_{s-3} and B_{s-12} are snake formers after irradiation like their parent, they show an increased resistance to crystal violet but not to furacin. A possible explanation would involve a combination of (a) differences in the efficiency with which different agents injure the mechanism of cell division, and (b) quantitative differences in whatever metabolic property is responsible for eventual recovery from injury to cell division. The results with B_{s-3} and B_{s-12} suggest that the order of decreasing efficiency may be u.v. radiation, furacin, crystal violet. This is supported by the observation that unlike *Escherichia coli* strain B, in *E. coli* strain s, selection for increased furacin resistance is not always equivalent to selection for increased u.v. resistance (Szybalski & Nelson, 1954; Woody-Karrer & Greenberg, 1963). Furthermore when strain s mutates to increased u.v. resistance, there may or may not be a reduction in snake formation (Curry & Greenberg, 1962). Thus an increased ability to recover from injury to cell division may not be the only cause of increased resistance to u.v. radiation. It would be interesting to know whether strain s is *hcr*⁻. If it is, then increased u.v. resistance could be due to mutation to *hcr*⁺. If it is already *hcr*⁺, the increased u.v. resistance of mutants which still form snakes after u.v. irradiation would suggest that there is a fourth mechanism of u.v. induced lethality in addition to those already mentioned. In any case, it is clear from the results with strain s and with the u.v. sensitive mutants of strain B that neither snake formation nor sensitivity to crystal violet and furacin are infallible indicators of u.v. sensitivity.

The diversity of B_s phenotypes reported here is not surprising since mutants with increased radio-resistance are also heterogeneous (Witkin, 1947; Woody-Karrer & Greenberg, 1963; Greenberg & Woody-Karrer, 1963). An interesting feature of the present results is that with the exception of B_{s-11} , all of the u.v. sensitive mutants differed from their B parent in more than one property. If failure to form snakes, crystal violet resistance and furacin resistance are considered as manifestations of a single property affecting cell division even though the magnitude of the change may vary so that all three aspects may not appear simultaneously, at least two properties changed in eleven of the twelve mutants. One change, either increased resistance to crystal violet alone or to both crystal violet and furacin is presumably related to a *decreased* u.v. sensitivity while the other, loss of ability to propagate u.v. irradiated T1 or some unknown change, presumably *increases* u.v. sensitivity.

The occurrence of multiple phenotypic changes as a result either of spontaneous mutation or of exposure to u.v. radiation is a fairly common phenomenon. In some cases, such as mutants of bacteriophage showing both a sensitivity to the presence of a suppressor gene in the host organism and an altered plaque morphology, there has been mutation at different loci (Campbell, 1961). The involvement of different genes is frequently the result of a deletion as for example in bacterial mutants exhibiting both resistance to T1 and a requirement for tryptophan (Yanofsky & Lennox, 1959). On the other hand, mutation at a single site can also cause multiple phenotypic changes. In some cases, the underlying common factor is known. Examples of this include bacterial mutation causing joint resistance to

bacteriophages T3, T7 and the unrelated T4 (the result of impaired production of the lipocarbohydrate portion of a bacterial surface site for adsorption) and bacteriophage mutation causing increased sensitivity to a suppressor gene in the host, to temperature and to pH extremes (the result of impaired production of an enzyme (Goebel & Jesaitis, 1952; Campbell, 1961)). It is also known that although allelic mutants show the same primary property governed by a gene, they may differ in other respects (Demerec, 1956). Since all of the *hcr*⁺ mutants except B_{s-11} show inhibitory concentrations of furacin within a narrow range and 'type C' crystal violet survival curves, the differences between them may be allelic. B_{s-3} and B_{s-12} may also be allelic mutants since they are both *hcr*⁻, both snake-formers and show the same response to crystal violet and furacin. In any case, decision as to which of the various possibilities of genetic change is responsible for each of the B_s phenotypes or groups of phenotypes must await the results of genetic analysis.

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