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INACTIVATION AND MUTAGENESIS BY PHOTOTOXINS USING *Escherichia coli* STRAINS DIFFERING IN SENSITIVITY TO NEAR- AND FAR-ULTRAVIOLET LIGHT

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Abstract—Four *Escherichia coli* strains carrying all the possible combinations of genes controlling sensitivity to near-UV (NUV; *nur* versus *nur*$^+$) and far-UV (FUV; *uvrA6* versus *uvrA*$^+$) were inactivated with broad-spectrum NUV together with specific phototoxins. The inactivation kinetics of the four strains are consistent with the previous reports that psoralen and angelicin inactivation is based on the formation of DNA adducts, while xanthotoxin (8-MOP) inactivation is based on the combined effects of DNA adduct formation and oxygen-dependent photodynamic action. At sufficiently high NUV fluences, xanthotoxol (8-HOP) induces lethal DNA lesions in an excision-deficient (*uvrA6*) strain. Inactivation by alpha-terthienyl plus NUV involves strictly membrane damage since the genes controlling the sensitivity to either NUV or FUV have no effect on inactivation kinetics. Using mutation to histidine independence (*his-4*$^+$) in the presence of NUV as a measure of mutagenicity by phototoxins, psoralen and xanthotoxin are mutagenic, angelicin is less mutagenic, and xanthotoxol and alpha-terthienyl are not mutagenic. None of the phototoxins tested in the presence of NUV were as mutagenic as FUV. Imperatorin and berberine were neither phototoxic nor mutagenic in this assay system. This assay thus provides a rapid qualitative screening procedure to identify the mode of action and mutagenicity of plant phototoxins with potential insecticidal properties.

Key Words—*Escherichia coli*, alpha-terthienyl, angelicin, xanthotoxin, xanthotoxol, psoralen, histidine independence, mutagenesis.

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INTRODUCTION

Over the past 20 years cellular systems in *Escherichia coli* have been identified for the repair of DNA damage whether it is induced by far-UV (FUV; 200–290 nm), ionizing radiation, or chemicals (for reviews see Witkin, 1976; Walker, 1984). The systems which have been most thoroughly characterized are the excision and recombination repair systems, both of which are inducible (Radman, 1974; Kenyon and Walker, 1981). Mutants defective in DNA repair have been identified principally based upon their hypersensitivity to inactivation by radiation or chemicals. If a strain carrying a mutation in a repair pathway (e.g., *recA*, recombinational repair defective, or *uvrA*, excision repair defective) is sensitive to inactivation by a particular treatment, it can be assumed that the damage induced by the treatment is at the DNA level and is reparable in corresponding nonmutant strains (for review see Scott et al., 1976).

In addition to DNA repair by the recombination and excision systems, there exists in *E. coli* cells another repair system specific for oxidative damage (Demple and Halbrook, 1983) resulting from treatment of cells with H_2O_2 or near-UV (NUV). However, it remains controversial as to whether this repair system is inducible (Sammartano and Tuveson, 1985). As is the case for the excision and recombinational repair systems, mutants have been identified representing defects in the pathway for the repair of oxidative damage. *E. coli* strains with *xthA* mutations (exonuclease III defective) are sensitive to inactivation by H_2O_2 (Demple et al., 1983) and broad-spectrum NUV (Sammartano and Tuveson, 1983). The fact that inactivation by NUV is strongly oxygen-dependent (Peak et al., 1983) suggests that the damage induced by these wavelengths is oxidative and probably involves the generation of H_2O_2.

Another gene (*nur*; NUV resistance) sensitizes cells specifically to inactivation by NUV (Tuveson and Jonas, 1979), photodynamic action (acridine orange plus visible light) and sunlight (Tuveson and March, 1980). Significantly, the *nur* mutation sensitizes *E. coli* to inactivation by NUV, photodynamic action (presumably oxidative), or sunlight, independent of whether the strain carries mutations in either the recombinational (*recA1* or *recA13*) or excision repair systems (*uvrA6*; Tuveson and Jonas, 1979; Tuveson, 1980). Strains with the *nur* mutation are sensitive to inactivation by H_2O_2 (Sammartano, Tuveson and Davenport, in preparation), suggesting that the mutation determines sensitivity to oxidative damage. The identification of mutations which specifically sensitize *E. coli* cells to oxidative damage allowed for the construction of a set of strains responding differentially to DNA damage which is oxidative (presumably DNA base damage; Massie et al., 1972) and nonoxidative DNA damage (such as photoadducts). In this paper, we show that these *E. coli* strains exhibited the inactivation kinetics expected for phototoxins whose mechanisms of action have been deduced previously. These strains may thus be useful in determining the inactivating mechanisms which underlie previously uncharacter-

ized phototoxins. The possible mutagenicity of such phototoxins can be assessed since this set of strains carries a mutable auxotrophic marker as well (*his-4*).

METHODS AND MATERIALS

Bacterial Strains. The strains RT7h, RT8h, RT9h, and RT10h were derived from strains RT7, RT8, RT9, and RT10 (Tuveson, 1980) by P1 transduction of a Tn10 insertion located next to the *his-4* allele in strain AB1157 (Leonardo et al., 1984). The four strains have the following properties in common: F^-, *argA21, lysA22, malA1, str 104,* $\lambda^r\lambda^-$, *supE44, zee::Tn10, his-4.* The strains differ genetically as follows: RT7h, *thi-1, nur, uvrA6;* RT8h, *thi-1*$^+$, *nur, uvrA*$^+$; RT9h, *thi-1, nur*$^+$ *uvrA6*; and RT10h, *thi-1*$^+$, *nur*$^+$, *uvrA*$^+$.

Media. The complex medium used was Luria-Bertani broth (LB; Miller, 1972). The semienriched minimal medium (SEM) used to assess survival consisted of appropriately supplemented minimal A medium (Miller, 1972) plus casamino acids (Difco, 0.4 ml of a 10% solution per liter; Kato et al., 1977) solidified with 1.5% Bacto-agar (Difco). To detect histidine independent (*his-4*$^+$) mutants, cells were spread on each of three SEM plates lacking histidine. To assess plate mutants (Webb, 1978), unirradiated cells were spread on minimal A medium lacking histidine (but containing arginine and lysine) and SEM lacking histidine. For the calculation of *his-4*$^+$ mutants per survivor, net *his-4*$^+$ mutants in a cell population were assessed as the total *his-4*$^+$ mutants minus the *his-4*$^+$ plate mutants.

Chemicals. Berberine, xanthotoxin (8-methoxypsoralen, 8-MOP), and psoralen were purchased from the Sigma Chemical Company, St. Louis, Missouri. Alpha-terthienyl (α-T) was a gift from J.T. Arnason, Biology Department, Ottawa University, Ottawa, Ontario, Canada. Imperatorin and angelicin were obtained from Roth-Chemie (Nahrstedt, Germany; now Atomergic, Plainview, New York) and HRI Associates (Emeryville, California), respectively. Xanthotoxol (8-hydroxypsoralen; 8-HOP) was synthesized by E. Heininger and J. Sternberg using a modification of the procedure of Schönberg and Sina (1950).

Broad-Spectrum NUV-Fluence Response Curves in Presence of Presumptive Phototoxins. Cells were grown at 37°C with shaking in side-arm flasks (Belco) containing 50 ml of LB broth (Miller, 1972). Growth was monitored by measuring the change in absorbance with a Klett-Summerson colorimeter equipped with a red filter. A 5-ml aliquot of stationary-phase cells (2.5 hr after entering the transition from exponential to stationary growth phase) was removed, washed three times with saline, and diluted to approximately 5.0×10^8 cells/ml in cold saline (ice bath temperature) and placed in a 16 × 160-mm tube with a magnetic flea at the bottom. Appropriately diluted presumptive phototoxin in 10 ml of 95% ethanol was added to the cell suspension following

which a 1-ml aliquot was withdrawn and held in the dark as a check on light-independent toxicity. Viability of the cells in the aliquot held in the dark was assessed after all manipulations had been completed with the NUV-treated suspension.

The broad-spectrum NUV source is identical to that described previously (Tuveson and Jonas, 1979). Briefly, the source consisted of a parallel array of four lamps (General Electric, 40 W BLB, integral filter) which emit radiation between 313 and 425 nm, with a maximum emission at 350 nm. The fluence rate was approximately 30 W/m^2 as estimated using a UVX Digital Radiometer (UVP, Inc., San Gabriel, California). This fluence rate is three times the estimate previously published which had been made with a Blak-Ray UV intensity meter (model J-227; Ultraviolet Products, Inc., San Gabriel, California) equipped with a J221 long-wave UV sensor (Tuveson and Jonas, 1979). The long-wave UV sensor proved to be defective.

Bioassay for Phototoxins. Ashwood-Smith et al. (1983) have described a bioassay procedure for the detection of furocoumarins and other photosensitizers. We have used essentially the same procedure described by Ashwood-Smith et al. (1983) for carrying out the bioassay procedures with our strains. Two thin-layer chromatographic (TLC) plates (silica gel 0.25 mm, glass backed, Merck, Darmstadt, Germany) were spotted with 20 μl of psoralen (1 mg/ml) and 20 μl of α-T (1 mg/ml). The plates were placed in contact with LB agar in each of four Petri dishes for 2 min. Essentially, the phototoxins were replicated onto each of four plates of LB agar. The individual plates in the set of four were overlayed with 4 ml of LB soft agar (0.8%) containing about 10^5 cells/ml of one of the four *E. coli* strains (RT7h–RT10h). One set of four plates was placed in the incubator immediately, while the second set of four was exposed to 43.7 kJ of broad-spectrum NUV before being placed in the incubator.

RESULTS AND DISCUSSION

The four strains (RT7h, RT8h, RT9h, and RT10h) which carry all four possible combinations of genes controlling excision proficiency (*uvrA6* versus $uvrA^+$) and sensitivity to oxidative (photodynamic) damage (*nur* versus nur^+) can be used to distinguish between effects of phototoxins which result from oxygen-independent DNA photoadduct formation and photodynamic damage which is oxygen dependent. To establish that this is the case, it was necessary to inactivate the four strains with phototoxins for which the mechanism of lethal action has been established in other ways (Scott et al., 1976). As expected, the strains carrying the *uvrA6* mutation (RT7h and RT8h) were sensitive to inactivation by psoralen plus NUV, supporting the established fact that psoralen phototoxicity is based on cycloadditions to DNA (Figure 1). When these same strains were tested with xanthotoxin (8-MOP), three of the four strains proved to be sensitive to inactivation (RT7h, RT8h, and RT9h; Figure 2). This is the

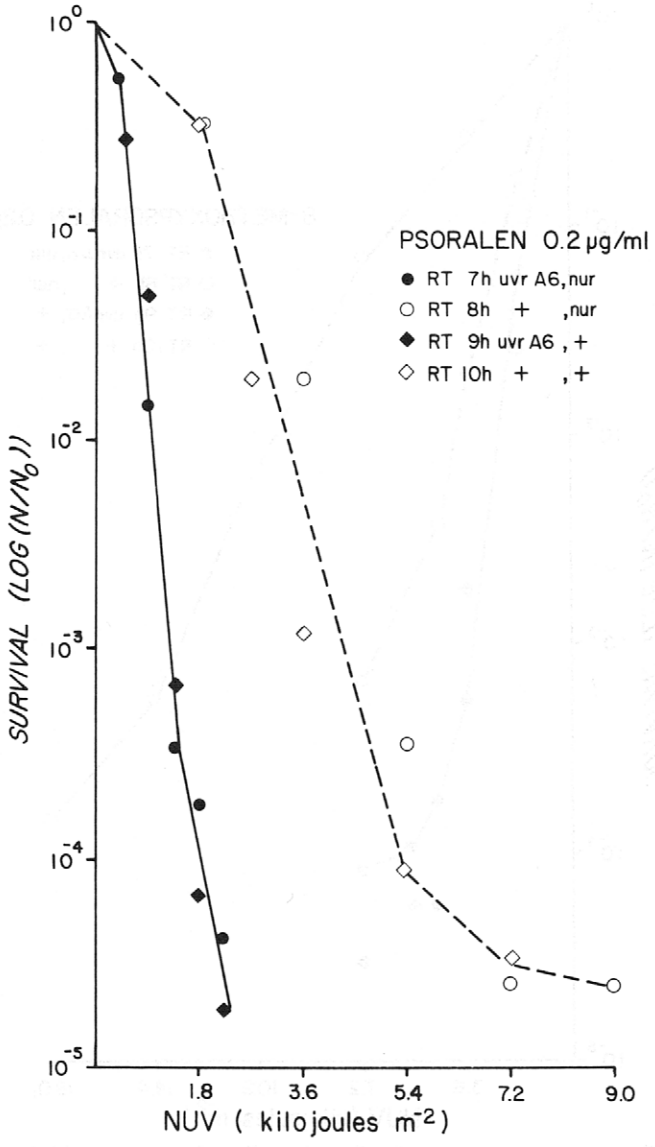


FIG. 1. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of psoralen.

result to be expected if xanthotoxin can act both as a classical photosensitizer and by DNA cycloadditions as has been suggested by Vedaldi et al. (1983). It should be noted that, although both psoralen and xanthotoxin were used at the rate of 0.2 µg/ml, the fluence of NUV required to obtain equivalent inactivation

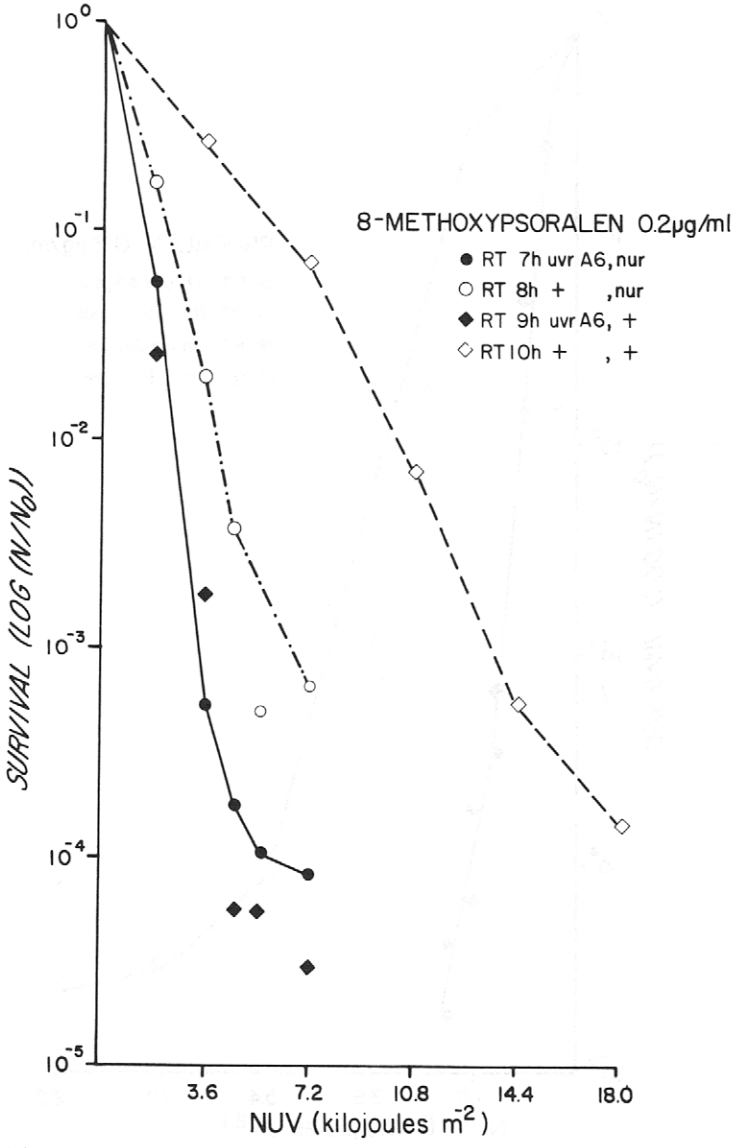


FIG. 2. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of 8-methoxypsoralen (xanthotoxin).

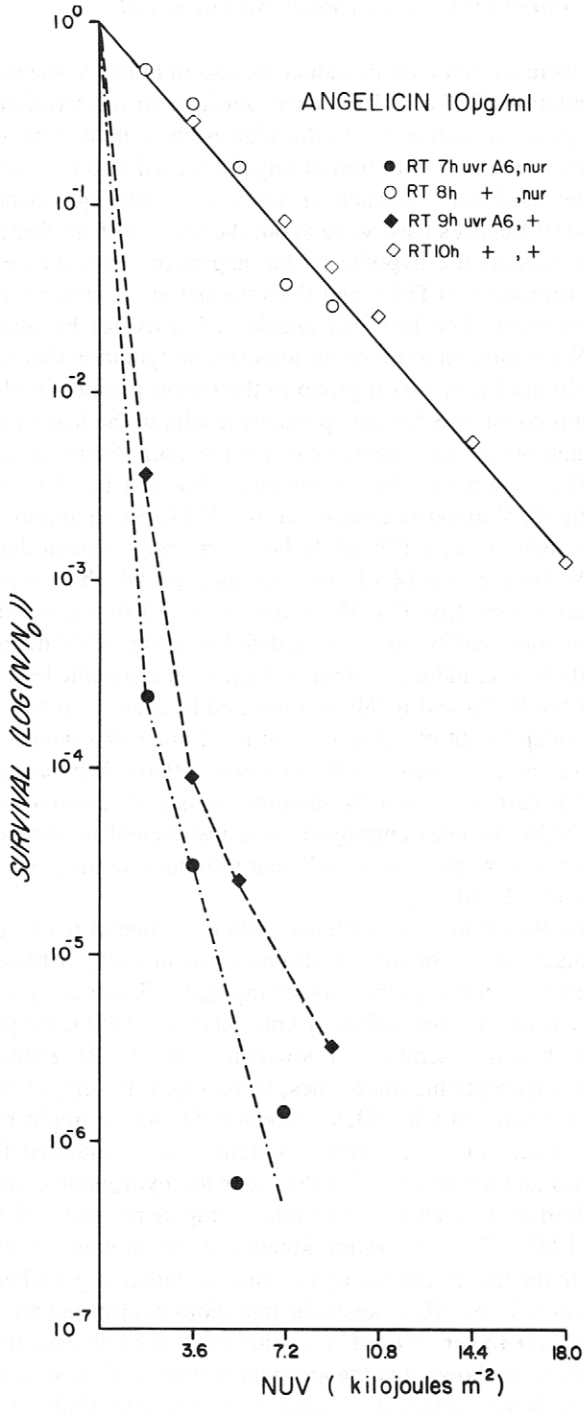
with xanthotoxin using the repair-proficient strain RT10h was greater than twice that required with psoralen. The reason for this difference is not apparent from these experiments since both chemicals produce cycloadditions to DNA.

When angelicin was used, the strains behaved as expected if its mode of

lethal action were based on monoadduct formation in DNA; the strains carrying the *uvrA6* mutation (RT7h, RT9h) were sensitive to inactivation by angelicin and NUV (Figure 3) (Scott et al., 1976). Consistent with the formation of monoadducts in DNA, the concentration of angelicin used in these experiments was five times that used with psoralen or xanthotoxin for equivalent toxicity, although the NUV fluences used were about the same with all three phototoxins. These results support the hypothesis that angelicin inactivates exclusively by monoadduct formation in DNA and does not act as a classical oxygen-dependent photosensitizer. The fact that angelicin inactivates by monoadduct formation in DNA is thought to reflect its angular configuration (Scott et al., 1976).

The addition of a hydroxyl group to the parent psoralen molecule at the 8 position (xanthotoxol or 8-hydroxyporalen) results in the loss of phototoxicity. We tested xanthotoxol as a phototoxin for the four strains used in our assay (RT7h–RT10h, Figure 4). The inactivation observed for three strains is that expected with NUV alone (Leonardo et al., 1984; Sammartano and Tuveson, 1984). The sensitivity of RT9h might be interpreted to mean that xanthotoxol at high NUV fluences (>18 kJ) induces damage which is reparable by the excision repair system (*uvrA*$^+$). However, in the RT7h strain, which also carries *uvrA6* mutation leading to excision deficiency, the NUV fluence tested was not sufficiently high to induce xanthotoxol excision-reparable lesions. The NUV sensitivity of the RT7h and RT8h is controlled by a gene (*nur*) which has been shown to be independent of either *recA* or *uvrA* mutations which sensitize cells to FUV (Tuveson and Jonas, 1979; Tuveson, 1980). We can speculate that, although RT7h carries the *uvrA6* mutation, it is not sensitive to xanthotoxol because the NUV fluences employed were insufficient to induce xanthotoxol lethal lesions but were more than sufficient to induce xanthotoxin lethal lesions (compare Figures 2 and 4).

When the four strains were treated with α-terthienyl (α-T) and NUV, the kinetics of inactivation for all the strains were indistinguishable (Figure 5). There is much evidence available suggesting that α-T acts as an oxygen-dependent (photodynamic) photosensitizer (Arnason et al., 1981), the principal lethal target for which is the membrane (Downum et al., 1982). Photochemical experiments with hydrophobic thiophenes, including α-T, suggest that these compounds, when irradiated with NUV, produce $^1O_2$ which might be expected to interact with membrane components (proteins and unsaturated fatty acids) to form peroxides and hydroperoxides that alter the hydrophobic character of the membrane, leading to leakage of cellular components and cell death (Reyftmann et al., 1985). The inactivation kinetics observed with our four strains are consistent with the membrane being the principal lethal target when *E. coli* cells are treated with α-T and NUV, since the mutations controlling NUV (*nur* versus *nur*$^+$) or FUV (*uvrA6* versus *uvrA*$^+$) sensitivity do not influence the inactivation kinetics. It has been shown that the *nur* gene sensitizes *E. coli* to photodynamic action when cells are treated with acridine orange plus visible light (Tuveson


ANGELICIN 10µg/ml
RT 7h uvr A6, nur
RT 8h + , nur
RT 9h uvr A6, +
RT 10h + , +
SURVIVAL (LOG(N/N0))
NUV (kilojoules m-2)
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
10^-7
3.6
7.2
10.8
14.4
18.0

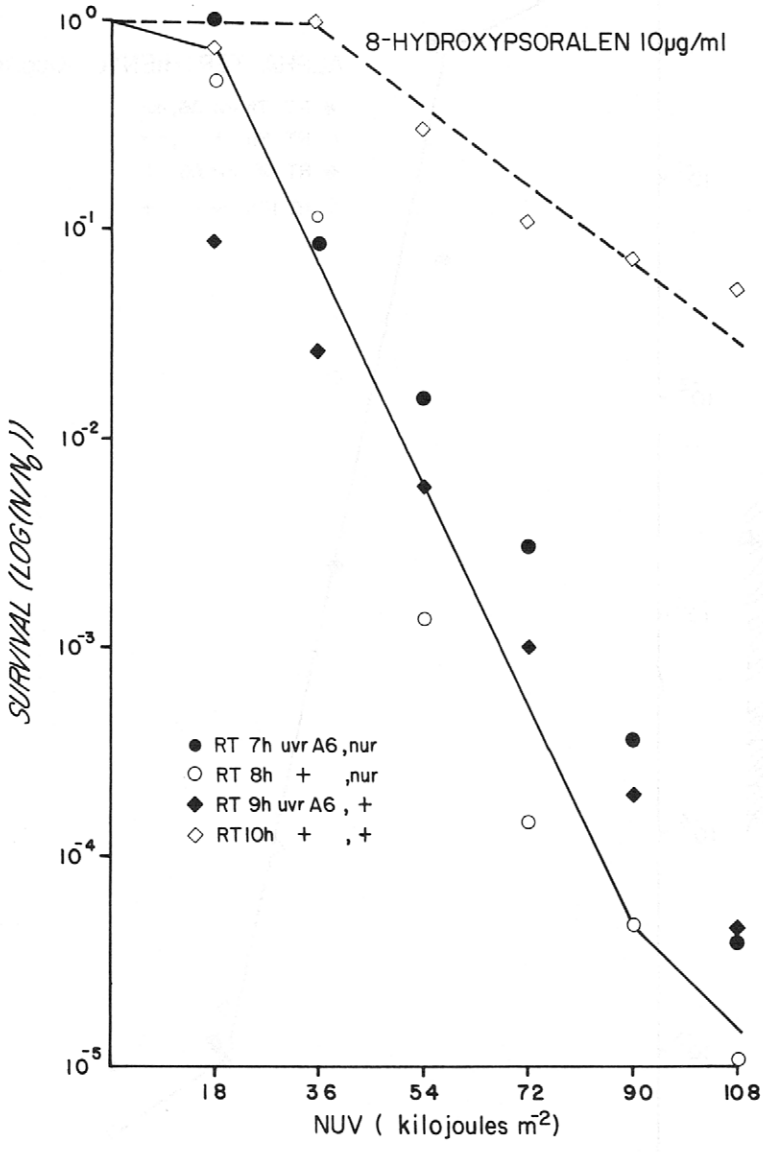


FIG. 4. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of 8-hydropsoralen (xanthotoxol).

FIG. 3. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of angelicin.

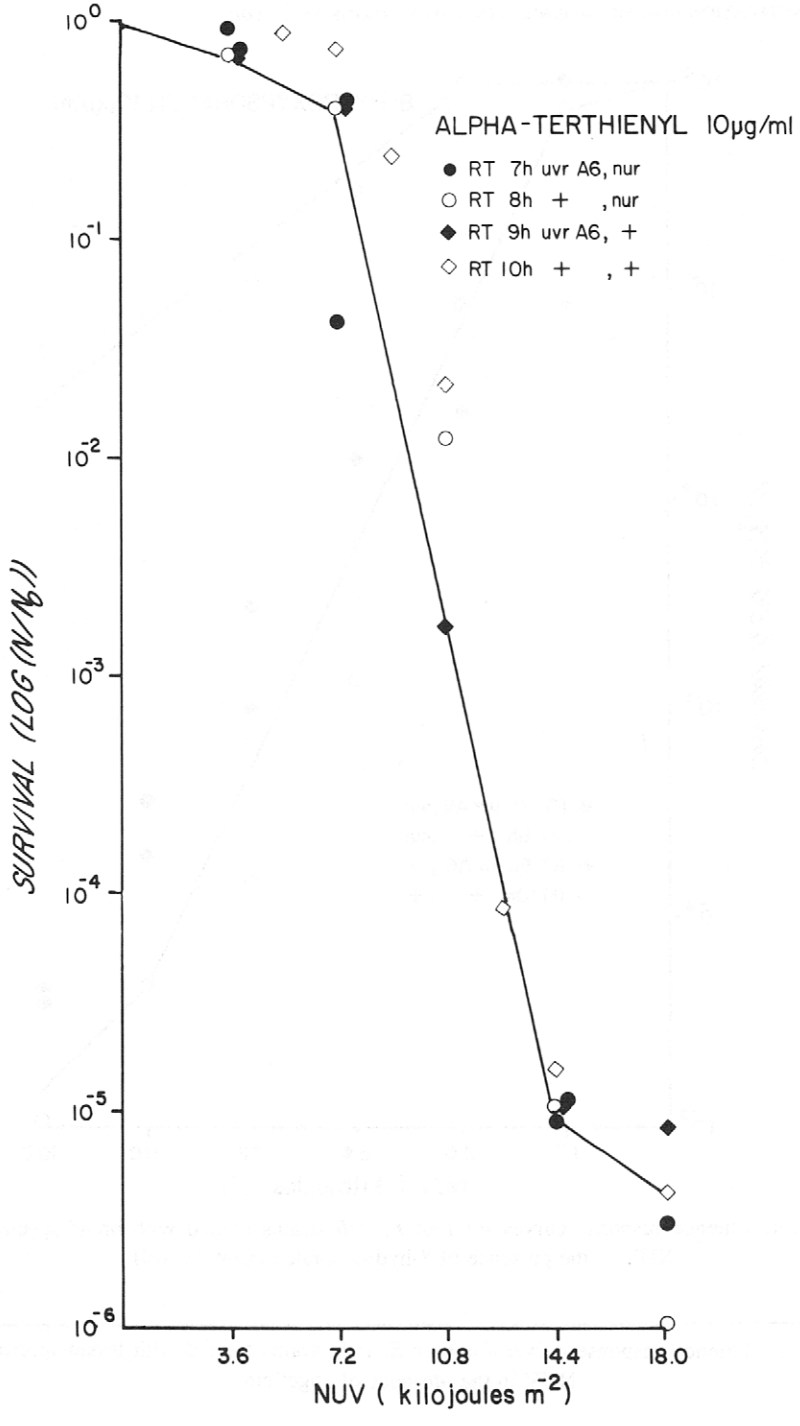

ALPHA-TERTHIENYL 10µg/ml
RT 7h uvr A6, nur
RT 8h + , nur
RT 9h uvr A6, +
RT 10h + , +
SURVIVAL (LOG (N/N0))
NUV (kilojoules m-2)
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
3.6
7.2
10.8
14.4
18.0

TABLE 1. PROPERTIES OF PHOTOTOXINS INVESTIGATED WITH *Escherichia coli* STRAINS RT7H, 8H, 9H, AND 10H

Phototoxin[a]	Mutagenicity	Concentration (μg/ml) used for inactivation experiments (conc./maximum conc. used)	F_{37} kilojoules for strain RT10h[a] (F_{37}/maximum F_{37})
Psoralen (Ps)	+	0.2 (0.02)	1.62 (0.03)
Xanthotoxin (8-methoxypsoralen, 8-MOP)	+	0.2 (0.02)	3.24 (0.60)
Angelicin (An)	+	10.0 (1.0)	2.70 (0.05)
Xanthotoxol (8-hydroxypsoralen, 8-HOP)	−	10.0 (1.0)	54.0 (1.0)
α-Terthienyl (α-T)	−	10.0 (1.0)	7.2 (0.13)

[a]Relative efficiency of inactivation: Ps > 8-MOP > α-T > An > 8-HOP.
[b]RT10h was used to compare F_{37} (fluence resulting in 37% survival) since it is repair proficient.

and March, 1980). This observation suggests that the *nur* gene sensitizes cells to oxidative damage involving DNA. When α-T is used as a photosensitizer, however, the *nur* gene has no sensitizing effect, as would be expected if the lethal effects of this compound plus NUV are restricted to the membrane.

When either imperatorin (Fahmy and Abu-Shady, 1947) or berberine (Philogène et al., 1984) were tested as possible phototoxins with the four *E. coli* strains RT7h, RT8h, RT9h, and RT10h, inactivation beyond that which could be accounted for by the NUV light system alone could not be demonstrated. In this assay system neither imperatorin nor berberine is phototoxic.

In all of the experiments just described (Figures 1–5), selection was carried out for histidine independence at the *his-4* locus. The comparison of the mutational results is difficult since the concentration of photosensitizer and the fluence of NUV used differs for each photosensitizer (Table 1). It seems most appropriate to compare the mutational results at equivalent survival levels assuming, therefore, the same number of lethal lesions are being produced by the phototoxins being compared. As a standard for mutagenicity, we elected to use FUV, which is highly mutagenic for the *his-4* allele carried by strains RT7h–RT10h (Kato et al., 1977). The comparison of the mutagenic effects of the various phototoxins tested and FUV are presented in Figure 6. It is apparent that psoralen, xanthotoxin (8-MOP), and angelicin are capable of inducing *his-4*$^+$ mutations but are not as efficient as FUV for the induction of such mutations. Mutations to histidine independence were not detected in the experiments in-

FIG. 5. Fluence–response curves for four *E. coli* strains treated with broad-spectrum NUV in the presence of α-terthienyl.

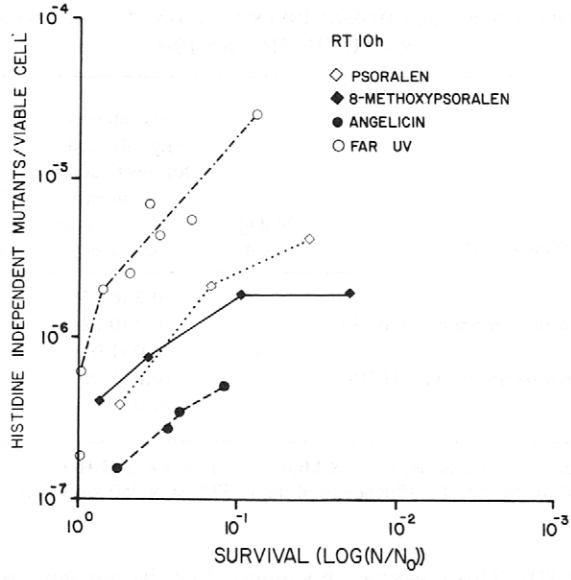


FIG. 6. Histidine-independent mutations per viable cells induced in strain RT10h with broad-spectrum NUV in the presence of phototoxins compared at equivalent survival levels.

volving xanthotoxol (8-HOP) or α-T. The mutation results with xanthotoxol were not unexpected since the inactivation observed with RT10h could be accounted for by the NUV radiation alone and NUV is not particularly mutagenic (Tyrrell, 1978; Leonardo et al., 1984). Even with RT9h, in which some fraction of the inactivation is the result of NUV and xanthotoxol, *his-4*$^+$ mutants were undetectable. The fact that *his-4*$^+$ mutants were not detectable with α-T plus NUV is what would be expected assuming the lethal target is the membrane rather than DNA.

In Table 1, the results are summarized and an evaluation of the relative efficiency of the phototoxins plus NUV for inactivation is presented. In this evaluation we have attempted to take into account the fact that the inactivation kinetics are determined by two variables: (1) the phototoxin concentration employed and (2) the fluence required to obtain 0.37 survival. As might have been expected, psoralen and xanthotoxin are the most efficient for inactivation and xanthotoxol the least efficient. In fact, the inactivation involving xanthotoxol is probably totally independent of the chemical and simply represents NUV inactivation.

The results reported here suggest that the four *E. coli* strains described (RT7h–RT10h) can be used to give a preliminary indication of the mechanism(s) by which a suspected phototoxin might inactivate cells. Furthermore, some indication of the mutagenicity of the presumptive phototoxin can be as-

sessed by selecting *his-4*$^+$ independent mutants in the same experiment in which inactivation kinetics are being assessed. Finally, identical inactivation kinetics for all four strains, as was seen with α-T (Figure 5), can be taken as preliminary evidence that the principal lethal target is the membrane.

Ashwood-Smith et al. (1983) have devised a method for assaying phototoxins involving transfer of presumptive phototoxins from TLC plates onto nutrient agar plates followed by overlaying the agar plates with soft agar (0.8%) containing bacteria (10^5–10^6 cells/ml). After the overlay solidifies, the plates are exposed to various NUV fluences. Where phototoxins were present on the TLC plate, a zone of inhibition will appear in the bacterial lawn on plates exposed to NUV before overnight incubation at 37°C. We ran an experiment to see whether similar procedures might be used with *E. coli* strains RT7h–RT10h. The results of this experiment are presented in Figure 7. It appears that the four strains described here can be used in a bioassay system comparable to that

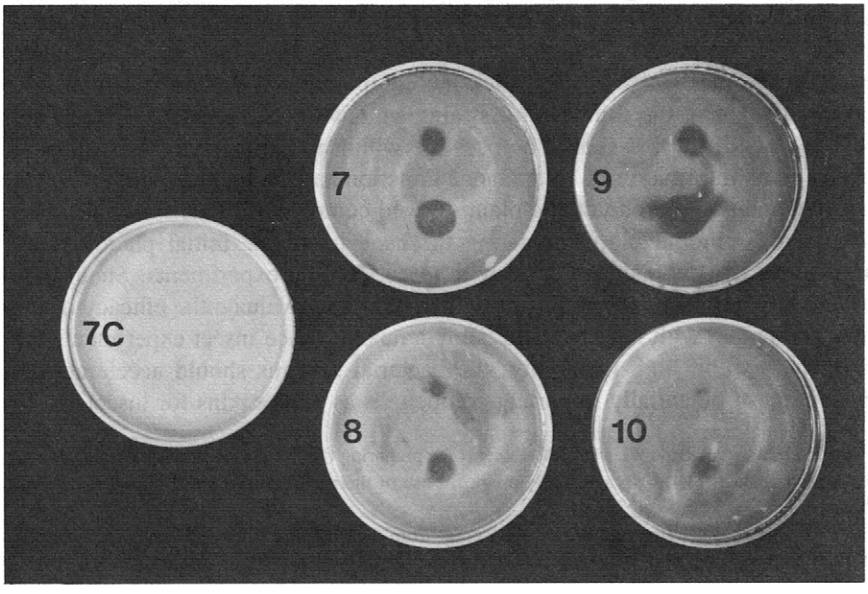


FIG. 7. Photograph of Petri dishes containing LB agar onto which TLC plates had been pressed for 2 min, following which a 4-ml overlay of LB agar seeded with indicator bacteria had been poured. 7 = RT7h, 8 = RT8h, 9 = RT9h, 10 = RT10h. Only a single unirradiated control is shown (7C = RT7h not exposed to broad-spectrum NUV). All other plates were exposed to broad-spectrum NUV. Upper clear zone in bacterial lawn = α-T; lower clear zone = psoralen.

described by Ashwood-Smith et al. (1983). The upper clear zone in the plates exposed to NUV marks the position of the α-T spot on the TLC plate and would be expected to be the same size for all four strains (Figure 5). However, R10h seems to be the least sensitive of the four strains to inactivation by α-T and NUV. The lower clear zone in the plates exposed to NUV marks the position of the psoralen spot on the TLC plate. Strains RT7h and RT9h exhibit larger zones of inactivation than do strains RT8h and RT10h, as expected, since these strains carry the *uvrA6* allele. These results imply that this qualitative bioassay can be used with the four strains described here to draw preliminary conclusions concerning the possible inactivation mechanism(s) characteristic of a particular phototoxin. However, firm conclusions concerning the inactivation mechanism(s) for such a phototoxin will require quantitative inactivation experiments.

The control of insect pests using synthetic chemicals is coming to a close. Alternative methods are needed to accomplish this objective. Clearly, there is not going to be any single method which will prove satisfactory under all circumstances. One possible method of control for insect pests would be to utilize the chemicals evolved by plants to control insect pests, such as phototoxins. These compounds, when ingested by insects feeding on plants, become toxic with exposure to sunlight. In principle, since these are "natural products," they are biodegradable and could be developed as ecologically sound "insecticides." To begin an evaluation of phototoxins, a rapid method is needed to evaluate the mechanism(s) by which the compound acts and its potential mutagenicity. Using the four *E. coli* strains we have constructed (RT7h, RT8h, RT9h, and RT10h), it should be possible within not more than two weeks to obtain some information as to the mode of action and the mutagenicity of newly isolated and purified phototoxic plant-derived compounds. This system is qualitative and reproducible, eliminating the need to do the initial phototoxicity assessment of new plant products with insect-feeding experiments. Should the bacterial experiments prove positive, then efforts to evaluate the efficacy of the new compound with insects could be undertaken. Since insect experiments are slow and exhibit high variability, the bacterial systems should accelerate the evaluation of potentially important compounds as phototoxins for insects.

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