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Role of Cloned Carotenoid Genes Expressed in *Escherichia coli* in Protecting against Inactivation by Near-UV Light and Specific Phototoxic Molecules

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Genes controlling carotenoid synthesis were cloned from *Erwinia herbicola* and expressed in an *Escherichia coli* strain. Carotenoids protect against high fluences of near-UV (NUV; 320 to 400 nm) but not against far-UV (200–300 nm). Protection of *E. coli* cells was not observed following treatment with either psoralen or 8-methoxypsoralen plus NUV. However, significant protection of cells producing carotenoids was observed with three photosensitizing molecules activated by NUV (*alpha*-terthienyl, harmine, and phenylheptatriyne) which are thought to have the membrane as an important lethal target. Protection of carotenoid-producing cells against inactivation was not observed with acridine orange plus visible light but was seen with toluidine blue O plus visible light.

Evidence that carotenoids function to protect microorganisms, plants, and animals against photodynamic action by quenching triplet-state photosensitizers (9, 12, 16), singlet oxygen, and radical oxygen species has been extensively reviewed (20, 21, 27). However, beta-carotene and perhaps carotenoids in general quench reactive oxygen species only under conditions of low oxygen tension (7). Dietary administration of carotenoids to hairless mice exhibiting induced porphyria can protect against porphyrin photosensitization induced by either UVA or UVB (28, 29).

Carotenoids in photosynthetic bacteria protect against photodynamic damage that probably involves chlorophyll as the endogenous photosensitizer (15). The protection observed in photosynthetic organisms probably results from direct quenching of the triplet state of chlorophyll as well as quenching of reactive-oxygen-related species (12). In the nonphotosynthetic bacterium *Myxococcus xanthus* that synthesizes carotenoids in stationary growth phase, protoporphyrin IX accumulates to high levels in stationary phase and probably serves as a potent endogenous photosensitizer (5, 6). Whether the formation of carotenoids by other nonphotosynthetic bacteria reflects the accumulation of an endogenous photosensitizer remains unknown (25, 26). Many phytopathogenic bacteria, such as *Erwinia herbicola* (33), synthesize yellow pigments, presumably carotenoids, which might serve to protect against reactive oxygen species resulting from absorption of visible light by chlorophyll present in the host plant.

Many fungi are known to produce carotenoids in abundance (14). Carotenoids protect *Neurospora crassa* against inactivation by specific exogenous dyes plus visible light (3, 38). Although Burchard and Dworkin (5) suggested that nonphotosynthetic organisms may synthesize carotenoids as protection against natural endogenous photosensitizers that accumulate in their cells, no natural endogenous photosensitizer has been identified in *Neurospora crassa*. However, it has been shown with the plant pathogenic fungus *Ustilago violacea* (19, 44) that certain mutants accumulate cytochrome *c*, sensitizing them to visible light. In addition, Mathews and Sistrom (26) demonstrated that a carotenoid-less mutant of the bacterium *Sarcina lutea* was more sensitive to inactivation by sunlight than was the wild type, consistent with the absorption of these wavelengths by some endogenous photosensitizer(s), perhaps a porphyrin-related molecule such as cytochrome *c*. Recent experiments with *Escherichia coli* have shown that porphyrin-related components can serve as endogenous photosensitizers (36).

Although *E. coli* does not normally synthesize pigments, Perry et al. (33) have succeeded in cloning and expressing genes involved in yellow pigmentation from *Erwinia herbicola* into *E. coli*. The chemical nature of the pigments was not reported. In this communication, we present evidence that these pigments are carotenoids.

Currently, there is interest in the targets attacked by and the mechanism(s) underlying inactivation by phototoxic molecules (2, 39, 40) as well as in the mechanisms by which organisms deal with reactive oxygen species (22). By using a series of *E. coli* strains differing in repair functions and catalase proficiency, it is possible to deduce the probable chemical mechanisms and cellular targets attacked by light-activated toxic molecules. In this paper, we describe the responses of an *E. coli* strain expressing carotenoids and equivalent strains not expressing carotenoids to a variety of inactivating agents, including specific phototoxic molecules. Our work suggests that these strains are of value in confirming results obtained with other *E. coli* strains designed to identify the lethal targets attacked by phototoxic molecules.

MATERIALS AND METHODS

The strain used was *E. coli* HB101 (23) (F$^-$ *hsdS20 recA13 ara14 proA2 lacY1 galK2 rspL20 xyl-5 mtl-1 supE44* λ^-. The plasmids used were pHC79 (33) (Ap^r cosmid) and pPL376 (33) [Ap^r; yellow pigment synthesis gene(s)].

The complex medium was Luria-Bertani (LB) containing 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 10 g of NaCl per liter (31).

The minimal medium (SEM) used to assess survival consisted of minimal A medium (31) supplemented with the

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nutritional requirements of *E. coli* HB101 (proline and leucine). In the experiments involving strain HB101 carrying a plasmid, ampicillin (50 μg ml^{-1}) was added to the minimal A agar plating medium as well as to the LB medium used to grow the strain in preparation for treatment.

Cells were grown at 37°C with shaking in sidearm flasks (Bellco Glass, Inc.) containing 50 ml of LB. Growth was monitored by measuring the change in absorbance with a Klett-Summerson colorimeter equipped with a green filter. A 5-ml sample of stationary-phase cells (2.5 h after entering the transition from exponential to stationary growth phase) was removed, washed three times with phosphate buffer (K-K; 0.067 M, pH 7.0), and diluted in cold buffer (ice bath temperature) to approximately 5.0×10^8 cells ml^{-1}. For treatment with far-UV (FUV), 10 ml of washed cell suspension was poured into a sterile petri dish (100 by 15 mm) and placed on a gently rotating platform shaker in a FUV chamber. Following appropriate intervals of irradiation, samples were withdrawn in preparation for plating to assess survival. All manipulations were carried out in a room equipped with Sylvania Gold (F40/GO) fluorescent lamps to prevent photoreactivation.

For treatment with near-UV (NUV), cells (10 ml) prepared exactly as described for FUV fluence-response experiments were placed in a test tube (16 by 160 mm) with a magnetic stirring bar at the bottom. In specific experiments, an appropriately diluted photosensitizer solution in 95% ethanol was added to the cell suspension to give the final desired concentration. A 1-ml sample was withdrawn and held in the dark as a check on light-independent toxicity. Viability of cells in the sample held in the dark was assessed after all manipulations were completed with the NUV-treated suspension.

Cells treated with visible light were prepared exactly as described for FUV treatment. The dye stock solutions (toluidine blue O [TB] and acridine orange [AO]) were prepared at 1.0 mg ml^{-1}, filter sterilized, and diluted appropriately into the cell suspension for irradiation. The cell suspension containing the dye was poured into a petri dish (100 by 15 mm) and placed on a bed of crushed ice under the visible light source. The ice was replaced periodically as melting occurred. The plate was removed from under the light source at appropriate intervals for sampling.

The FUV chamber was identical to the one described previously (41), consisting of a bank of six General Electric germicidal lamps which emit 86% of their radiant power at 253.7 nm. The fluence rate was measured with a DIX-100X digital radiometer equipped with a DIX-254 sensor (Spectroline) and found to be 0.7 W m^{-2}.

The broad-spectrum NUV source used for the bacterial experiments was identical to that used in all our previous experiments involving broad-spectrum NUV (41). It consists of a bank of four lamps (GE40BLB; integral filter) with a range of emission from 300 to 425 nm, with a maximum emission at 350 nm. The source was maintained in a cold room at 10°C to reduce heating effects. Sham NUV irradiation of foil-wrapped cell suspensions showed no inactivation (41). When measured with a DRC-100 digital radiometer equipped with a DIX-365 sensor (Spectroline), the fluence rate ranged from 20 to 24 W m^{-2} during the course of these experiments.

Cells treated with visible light were irradiated with a Sylvania 1,000-W metal halide bulb. The radiation passed through 21 cm of water contained in a stainless steel tank with a plate glass bottom. The water circulated and was maintained at about 20°C. The system is identical to that used in previous experiments (42). At the level of the cell suspension, the irradiance was 1,350 microeinsteins m^{-2} s^{-1} (~1.41 W m^{-2}) of photosynthetically active radiation as measured with a LI-COR Quantum/Radiometer/Photometer (model LI-185B) equipped with a Quantum Sensor.

An overnight culture of *E. coli* cells (45 ml) was centrifuged; the medium was poured off, and the cells were washed once with phosphate buffer (0.067 M phosphate [K-K], pH 7.0). Following the washing, the cells were again centrifuged, and the resulting pellet was suspended in an acetone-methanol mixture (7:2, vol/vol). The resulting slurry was centrifuged, and the supernatant was retained for the spectrophotometric characterization of the pigments. UV visible spectra were determined with a Perkin-Elmer double-beam spectrophotometer (model 552A).

RESULTS AND DISCUSSION

Strains HB101, HB101(pHC79), and HB101(pPL376) were grown to stationary phase in LB broth and extracted to test for carotenoids. The yellow-pigmented strain HB101 (pPL376) was also grown in LB broth plus glucose since Perry et al. (33) have reported that pigmentation appears to be under catabolite repression. The UV visible spectrum of the lipid extract of strain HB101(pPL376) grown in the presence of glucose displayed a carotenoid pattern in the visible region (λ_{max}, 453 and 478 nm; shoulder at 425 nm) unique to oxygenated xanthophylls such as fucoxanthin. A small peak (λ_{max}, 333 nm) was observed in the NUV. The same pattern of absorption peaks was observed in the extract from HB101(pPL376) grown without glucose under about 10% of the left intensity. Comparable extracts from strains HB101 and HB101(pHC79) showed no absorption peaks in the NUV or visible regions.

Thin-layer chromatography (5:1 $CHCl_3$-isopropanol; silica gel) of the lipid extract of strain HB101(pPL376) and visualization of the plates by FUV (254 nm) and I_2 revealed six spots, four of which were present in the HB101 extract. The only spot with visible yellow color was at the origin. No detectable beta-carotene was present. The two spots from HB101(pPL376) that were not present in HB101 ran at R_f values of 0.85 and 0.27 and may have been due to oxygenated carotenoids. Results of high-pressure liquid chromatographic analyses of the yellow pigments from strain HB101(pPL376) revealed three peaks exhibiting spectra consistent with beta-carotene, but these compounds were more polar than beta-carotene. The precise identification of these compounds awaits the accumulation of sufficient material for mass spectral analyses (H.-C. B. Yen, personal communication), but there is little doubt that the pigments being expressed in *E. coli* HB101 and derived from *Erwinia herbicola* are in fact carotenoids.

Three *E. coli* strains were used to investigate protection by carotenoids from a variety of inactivating agents: HB101, the recipient strain used for transformation with the cloning vector; HB101(pHC79), the recipient strain carrying the plasmid into which the carotenoid genes were cloned; and HB101(pPL376), the recipient strain carrying the plasmid into which the carotenoid genes were cloned and expressing the cloned pigment genes. When these strains were treated with FUV, the kinetics of inactivation were indistinguishable (data not shown). This result is consistent with results reported with *N. crassa* in which the inactivation kinetics of wild-type (carotenoid-containing) and albino (carotenoid-less) conidia following treatment with FUV were essentially identical (3). Since the lethal target for FUV is DNA,

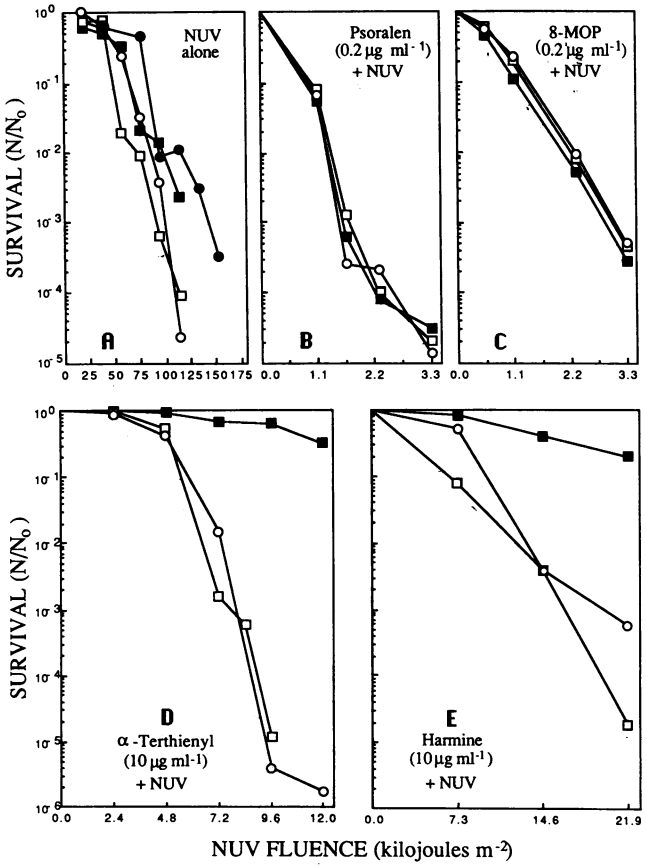

FIG. 1. Fluence-response curves. Symbols: ○, HB101; □, HB101(pHC79); ●, HB101(pPL376) experiment 1; ■, HB101 (pPL376) experiment 2. 8-MOP, 8-Methoxypsoralen.

protection from FUV by carotenoids is not expected because carotenoids are associated with the cell membrane in bacteria (20). Using *E. coli* strains equivalent to those used here, Perry et al. (33) reported that the pigmented and nonpigmented strains of *E. coli* are equally sensitive to inactivation by UV. Since the wavelengths were not specified, we assume that these investigators used FUV.

When the three *E. coli* strains were treated with NUV, the kinetics of inactivation for strains HB101 and HB101 (pHC79) were essentially identical (Fig. 1A). With the carotenoid-producing strain, the results were different in that there was evidence of protection by carotenoids at light intensities above 70 kJ m^{-2}. When the experiment was repeated, the same general result was obtained; the inactivation curve was biphasic, but in the second experiment the initial shoulder was extended. Because the inactivation kinetics of the carotenoid-producing strain were unique in our experience, we repeated the inactivation experiments with strain HB101(pPL376) and one of the carotenoidless strains [HB101(pHC79)] using new NUV bulbs producing a fluence twice that of the original bulbs. The inactivation kinetics for the carotenoid-producing strain were again complex (biphasic; two experiments), while those of the carotenoidless strain [HB101(pHC79); single experiment] again exhibited a shoulder followed by a final exponential slope (data not shown). We have no real explanation for the complex character of the inactivation curve obtained with the carotenoid-producing strain [HB101(pPL376)], but we suggest that it may reflect the fact that both DNA and the membrane are important lethal targets for NUV as has been previously suggested (11, 32). Previous results with a *N. crassa* carotenoid-producing strain and a carotenoidless mutant have shown that carotenoids offer only limited protection against the inactivating effects of NUV alone (3, 38). The fact that carotenoids offer limited protection against NUV inactivation in both *E. coli* and *N. crassa* may mean that one important protective role for carotenoids is against photosensitized inactivation involving endogenous or exogenous sensitizers and visible light (5, 6, 19, 44). The chief endogenous photosensitizing molecules for visible light are probably porphyrin-related materials, such as chlorophyll in plants, protoporphyrin IX in *M. xanthus* (4, 5), and cytochrome *c* in certain fungi, such as *U. violacea* (19, 44). However, it is also possible that the endogenous photosensitizers for NUV in both *E. coli* and *N. crassa* produce cytotoxic agents which are quenched to only a limited extent by carotenoids.

The carotenoidless and carotenoid-producing *E. coli* strains were tested with a variety of photosensitizing molecules activated by NUV. The maximum NUV fluence used to activate the phototoxins resulted in no inactivation of the three *E. coli* strains when used alone (compare Fig. 1A with Fig. 1B through E and Fig. 2A).

Psoralen is a phototoxin that acts by forming cycloadducts to DNA (24, 37), while 8-methoxypsoralen acts both by cycloaddition to DNA and as a classical oxygen-dependent photosensitizer (43). These two phototoxic molecules were used to inactivate the *E. coli* strain expressing carotenoids as well as strains not expressing carotenoids (Fig. 1B and C). These results demonstrate that carotenoids did not protect against these phototoxins. This may mean that either (i) cycloadditions to DNA so strongly predominate that the inactivation assay may not be sensitive enough to detect damage involving the membrane as a lethal target or (ii) the activated oxygen species generated by 8-methoxypsoralen plus NUV may not be generated in the vicinity of the membrane (20).

Numerous investigations have shown that alpha-terthienyl (alpha-T) plus NUV acts as an oxygen-dependent photosensitizer (1, 34) whose principal lethal target is the membrane (10). As expected, carotenoids strongly protected *E. coli* against inactivation by alpha-T plus NUV (Fig. 1D).

The protective effect of carotenoids in *E. coli* was tested with two additional phototoxic molecules activated by NUV. Harmine is a beta-carboline alkaloid with photoactivated antiviral activity (17). It is apparent that carotenoids offered substantial protection against inactivation by harmine plus NUV (Fig. 1E). The protection was nearly as significant as that offered by carotenoids against inactivation by alpha-T plus NUV (compare Fig. 1D and E). These results may mean that the membrane is an important lethal target for attack by harmine plus NUV in *E. coli*. The results of other experiments with a series of *E. coli* strains designed to test the possible mechanism(s) of inactivation by phototoxic molecules are consistent with this conclusion (39, 40).

Phenylheptatriyne (PHT) belongs to a large group of polyacetylenes and related thiophene derivatives found in the plant family Compositae that exhibits strong oxygen-dependent phototoxicity (8, 13, 30). The carotenoid-producing strain [HB101(pPL376); Fig. 2A], was found to be resistant when compared with either the HB101 or the plasmid-containing strain [[HB101(pHC79)]. However, it should be noted that the plasmid (pHC79) sensitizes HB101 to inactivation by PHT plus NUV. The presence of a plasmid (pBR322) has been shown to sensitize *E. coli* to inactivation

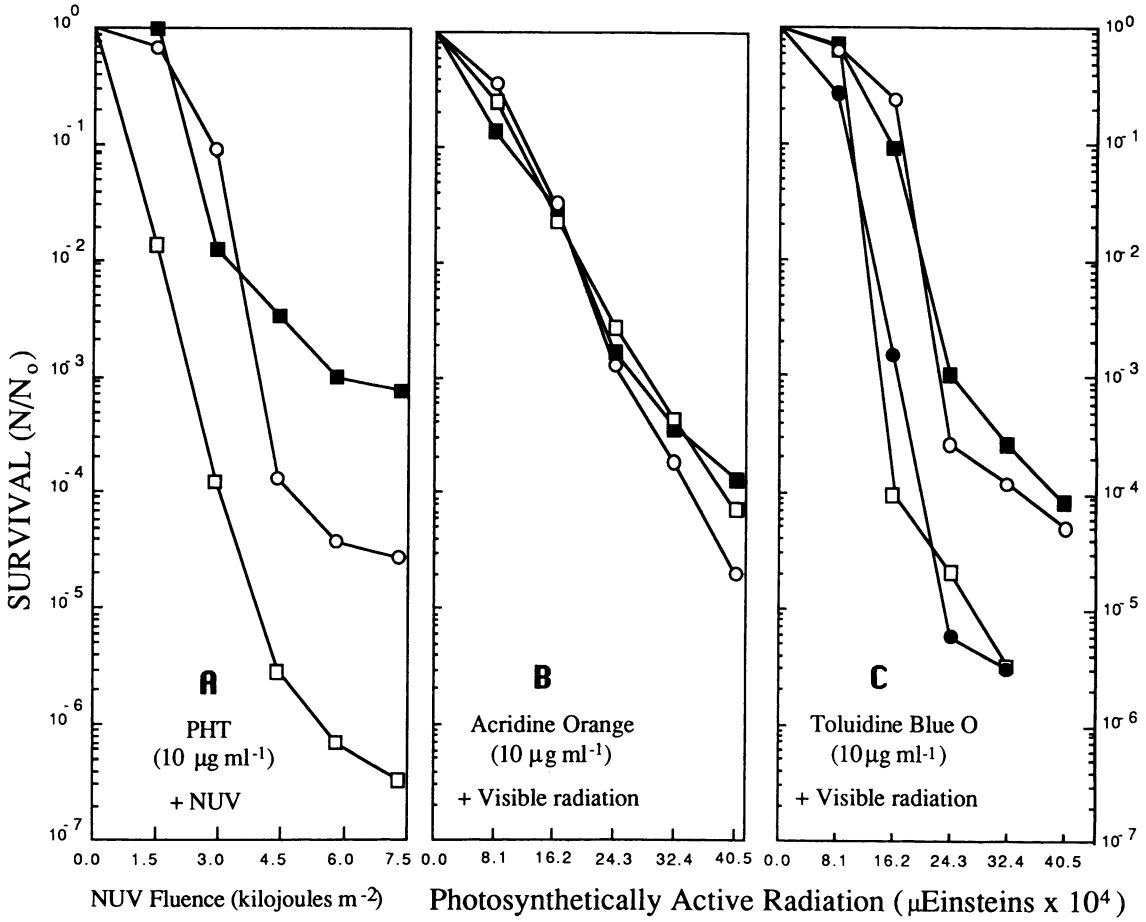

FIG. 2. Fluence-response curves. Symbols: ○, HB101; □, HB101(pHC79) experiment 1; ●, HB101(pHC79) experiment 2; ■, HB101 (pPL376).

by NUV alone (36). Because pBR322 carried the tetracycline resistance (Tc^r) marker, it was hypothesized that the plasmid might sensitize the recipient strain GR84N to NUV since tetracycline resistance is based on a modification of the membrane. If this were true either the complete or partial deletion of Tc^r would be expected to restore the resistance of strain GR84N to NUV inactivation. In fact the resistance to NUV was only partially restored by the complete or partial deletion of the Tc^r gene (36). Therefore, some other function controlled by the plasmid must contribute to NUV sensitivity. Since the plasmid pHC79 does not carry a Tc^r marker, the sensitization of HB101 to inactivation by PHT plus NUV must be related to some other unidentified function controlled by the plasmid, analogous to the sensitization of *E. coli* GR84N to NUV inactivation by the Tc^r-deleted plasmid derived from pBR322 (36).

The protection offered by carotenoids to inactivation by PHT plus NUV is not comparable with that seen with alpha-T plus NUV (compare Fig. 1D and 2A). When strain HB101(pHC79) was inactivated to about 10^{-5} by PHT plus NUV, the survival of the carotenoid-producing strain HB101(pPL376) was reduced to slightly less than 10^{-2}. In contrast, following treatment with alpha-T plus NUV, the survival of the carotenoid-producing strain HB101(pPL376) was reduced by about 50% at a point at which the survival of the carotenoidless strains had been reduced to 10^{-5}. This difference in the protection offered by carotenoids to inactivation by PHT plus NUV versus alpha-T plus NUV may mean that the membrane is a relatively more important lethal target for alpha-T than for PHT or that PHT and alpha-T have different membrane targets differentially protected by carotenoids.

Protection by carotenoids against inactivation by two dyes activated by visible light was also tested. Since AO binds to DNA (18, 20), carotenoids were not expected to protect against this dye when activated by visible light, and this expectation was met (Fig. 2B). The results are consistent with those of Roth (35), who demonstrated that a carotenoidless mutant of *Sarcina lutea* treated with AO plus visible light was no more sensitive to inactivation than a wild-type strain.

When the same experiments were run with TB, some protection against inactivation was observed (Fig. 2C). Reported not to enter yeast cells, TB presumably photoinactivates them by generating reactive oxygen species at the yeast cell surface, leading to lethal membrane damage (18). In addition, Mathews (24) suggested that the membrane is an important lethal target for TB plus visible light in the bacterium *S. lutea*. The results shown in Fig. 2C suggest that the presence of a plasmid (pHC79) sensitizes HB101 cells to inactivation by TB plus visible light, as was observed with PHT plus NUV (Fig. 2A). HB101 and the carotenoid-producing strain HB101(pPL376) are equivalent in their sensitivity to inactivation by TB plus visible light (Fig. 2C). If the valid comparison is between the two plasmid-containing strains, the protection by carotenoids from inactiva-

tion by TB plus visible light is different from that observed with either alpha-T or harmine plus NUV (compare Fig. 2C with Fig. 1D and E).

As was true for NUV, control experiments revealed that treatment of the three *E. coli* strains used in these investigations with visible light alone resulted in no detectable inactivation. Thus, the simultaneous presence of dye plus visible light was necessary to detect significant inactivation.

The experiments reported here demonstrate that genes controlling carotenoid synthesis and expressed in *E. coli* offer protection against inactivating agents shown by others to have the membrane as an important lethal target (alpha-T and PHT plus NUV) while being without effect for agents having lethal targets other than the membrane (FUV and AO plus visible light). The strains used in these investigations can serve as useful supplements to strains recently developed to assess the possible mechanism(s) by which phototoxic molecules inactivates cells (39, 40). The carotenoid-producing strain HB101(pPL376) might be expected to be resistant to inactivation when compared with the carotenoid-less strains for any phototoxic molecules demonstrated to have the membrane as an important lethal target based on tests with other *E. coli* strains.

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