mutant tissue and be analyzed by DNA blot analysis. If the cloned sequence is part of the gene of interest, a length polymorphism indistinguishable from the progenitor allele will be observed in a DNA blot analysis of DNA from germinal or somatic revertant tissue while a length polymorphism associated with the transposon-induced allele is present in DNA isolated from the nonrevertant tissue. These data would demonstrate that the cloned DNA sequence is part of the gene of interest.

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[28] Protection by Cloned Carotenoid Genes Expressed in *Escherichia coli* against Phototoxic Molecules Activated by Near-Ultraviolet Light

By R. W. TUVESON and G. SANDMANN

Introduction

The evidence that carotenoids protect plants, animals, and microorganisms against photodynamic action by quenching triplet state photosensitizers, singlet oxygen ($^1O_2$), and perhaps radical oxygen species has been reviewed.[1-3] Photosynthetic bacteria contain carotenoids that serve to protect against photodynamic action probably involving chlorophyll as an endogenous photosynthesizer.[4] In the nonphotosynthetic bacterium *Myxococcus xanthus,* protoporphyrin IX accumulates to high levels when the cells enter stationary phase. Concomitant with the accumulation of protoporphyrin IX, carotenoids are synthesized probably to protect against photodynamic action resulting from protoporphyrin IX serving as an en-

[1] N. I. Krinsky, *in* "Photophysiology—Current Topics" (A. C. Giese, ed.), p. 123. Academic Press, New York and London, 1968.
[2] N. I. Krinsky, *in* "Carotenoids" (O. Isler, H. Gutman, and U. Solms, eds.), p. 669. Birkhauser, Basel and Stuttgart, 1971.
[3] M. M. Mathews-Roth, *Photochem. Photobiol.* **40,** 63 (1984).
[4] M. Griffths, M. R. Sistrom, and G. Cohen-Bazire, *Nature (London)* **176,** 1211 (1955).

dogenous photosensitizer.[5,6] Epiphytic and phytopathogenic bacteria associated with aboveground plant parts (e.g., *Erwinia herbicola*[7] frequently produce yellow pigments, presumably carotenoids, to protect against photodynamic damage that may result from the absorption of visible light by the chlorophyll of the host plant acting as a photosensitizer. Many fungi produce carotenoids, and in the case of *Neurospora crassa* these pigments have been shown to protect against photodynamic action involving specific exogenous dyes.[8-10] Because *N. crassa* as well as many other fungi are exposed to sunlight in nature, it is plausible that the carotenoids serve to protect against light-induced damage at the wavelengths being absorbed by endogenous photosensitizers such as cytochromes or flavins.[11,12] Normally *Escherichia coli* does not synthesize carotenoids, but Perry *et al.*[7] were able to clone into *E. coli* a 15.6-kb DNA fragment from an epiphytic bacterium, *E. herbicola,* carrying genes allowing for the synthesis of the yellow pigments characteristic of this species. The carotenoid pigments expressed in *E. coli* strain LE392(pPL376) have been identified as phytoene (15-*cis* isomer), β-carotene (all-*trans,* 9-*cis,* and 15-*cis*), β-cryptoxanthin (3-hydroxy-β-carotene), zeaxanthin (3,3′-dihydroxy-β-carotene), and the corresponding carotenoid glycosides.[13] The cloned DNA fragment specifying the genes controlling the biosynthesis of the carotenoids characteristic of *E. herbicola* and expressed in *E. coli* has been partially sequenced.[14] Based on analogy with DNA sequences from *Rhodobacter capsulatus,* it has been suggested that the sequences derived from *E. herbicola* carry information for three enzymes involved in the early steps in the biosynthesis of carotenoid pigments [prephytoene pyrophosphate synthase (CrtB), phytoene synthase (CrtE), and phytoene dehydrogenase (CrtI)] in addition to other as yet unidentified gene products. The idea that the early genes concerned with carotenoid biosynthesis are highly conserved is supported by the observation that the deduced amino acid sequences for phytoene dehydrogenase from *N. crassa* and *R. capsulatus* coded for by the *al-1* and

[5] R. P. Burchard and M. Dworkin, *J. Bacteriol.* **91,** 535 (1966).
[6] R. P. Burchard, S. A. Gordon, and M. Dworkin, *J. Bacteriol.* **91,** 896 (1966).
[7] K. L. Perry, T. A. Simonitch, K. J. Harrison-Lavoie, and S.-T. Liu, *J. Bacteriol.* **168,** 607 (1986).
[8] T. W. Goodwin, *Prog. Ind. Microbiol.* **11,** 31 (1972).
[9] P. L. Blanc, R. W. Tuveson, and M. L. Sargent, *J. Bacteriol.* **125,** 616 (1976).
[10] S. A. Thomas, M. L. Sargent, and R. W. Tuveson, *Photochem. Photobiol.* **33,** 349 (1981).
[11] R. W. Tuveson and L. J. Sammartano, *Photochem. Photobiol.* **43,** 621 (1986).
[12] R. E. Lloyd, J. L. Rinkenberger, B. A. Hug, and R. W. Tuveson, *Photochem. Photobiol.* **52,** 897 (1990).
[13] G. Sandmann, W. S. Woods, and R. W. Tuveson, *FEMS Microbiol. Lett.* **71,** 77 (1990).
[14] G. A. Armstrong, M. Alberti, and J. E. Hearst, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 9975 (1990).

crtI genes, respectively, are approximately 30% identical.[15] The *E. coli* strains carrying and expressing genes for carotenoid biosynthesis are useful for assessing the protection offered by carotenoids to a variety of photosensitizers as well as near-ultraviolet light (near-UV; 320–400 nm) along.[16,17]

Methods for Using *Escherichia coli* to Assess Protection by Carotenoids

Two *E. coli* strains (HB101; LE392) have been transformed with three plasmids: (1) pHC79, the original plasmid into which the *E. herbicola* carotenoid biosynthetic genes were cloned; (2) pPL376, a derivative of pHC79 carrying genes necessary for carotenoid biosynthesis; and (3) pdel16, a derivative of pPL376 from which a 1.9-kb *Ava*I fragment has been deleted leading to the accumulation of lycopene in *E. coli* strains carrying this plasmid (probably equivalent to the plasmid originally described as pHL545[7]).

The recipient and plasmid-carrying strains are grown in Luria–Bertani (LB) medium containing 10 g tryptone (Difco, Detroit, MI) 5 g yeast extract (Difco), and 10 g NaCl per liter.[18] Cells are grown at 37° with vigorous shaking (300 rpm) in side-arm flasks (Bellco, Vineland, NJ) containing either 10 or 50 ml of LB to which is added 50 μg/ml ampicillin for the plasmid-containing strains. Vigorous shaking is essential since carotenoid biosynthesis is strongly oxygen dependent. Growth is monitored by measuring the change in absorbance with a Klett–Summerson colorimeter equipped with a green filter. Stationary phase cells must be used since the synthesis of carotenoids does not occur until well into stationary phase (usually 2.5 hr after entering the transition from exponential to stationary growth phase). A 5-ml sample of cells is removed, washed 3 times with 67 m*M*, pH 7.0, phosphate buffer (K-K), diluted in cold buffer (ice bath temperature) to approximately 5.0×10^8 cells/ml, and placed in a 16×160 mm test tube with a magnetic stirring bar at the bottom. For strains expected to produce carotenoids, carotenoid synthesis can be recognized by the obvious pigmentation of the bacterial pellets following centrifugation (bright yellow for strains carrying plasmid pPL376; pink for strains carrying plasmid pdel 16). In experiments involving photosensitiz-

[15] T. J. Schmidhauser, F. R. Lauter, V. E. A. Russo, and C. Yanofsky, *Mol. Cell. Biol.* **10,** 5064 (1990).

[16] R. W. Tuveson, R. A. Larson, and J. Kagan, *J. Bacteriol.* **170,** 4675 (1988).

[17] R. W. Tuveson, G. R. Wang, T. P. Wang, and J. Kagan, *Photochem. Photobiol.* **52,** 993 (1990).

[18] J. H. Miller, "Experiments in Molecular Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972.

ing molecules, the chemicals are dissolved in either 95% ethanol or dimethyl sulfoxide (DMSO) and added to the cell suspension to give the final desired concentration. A 1-ml sample is withdrawn and held in the dark as a check on light-independent toxicity. Viability of cells in this sample is assessed after all manipulations are completed with the near-UV-treated suspension.

The minimal medium (SEM) used to assess viability consists of minimal A medium[18] supplemented with the nutritional requirements of the particular *E. coli* cloning strain (HB101, proline and leucine; LE392, methionine). In experiments involving plasmid-carrying strains, ampicillin (50 μg/ml) is added to the minimal A agar plating medium.

The broad-spectrum near-UV source used for assessing protection by carotenoids is identical to that used in all our previous experiments involving broad-spectrum near-UV.[19] It consists of a bank of four lamps (GE40BLB, integral filter) emitting between 300 and 425 nm, with maximum intensity at 350 nm. The source is maintained in a cold room at 10° to reduce heating effects. Sham near-UV irradiation of foil-wrapped cell suspensions show no inactivation.[19] When measured with a DRC-100 Digital Radiometer equipped with a DIX-365 sensor (Spectroline), the fluence rate ranges from 40 to 43 J sec^{-1} m^{-2} during the course of the experiments described here.

Carotenoid Protection

In previous experiments,[13,16] six *E. coli* strains were used to investigate carotenoid protection from a variety of inactivating agents. In those experiments, the recipient strains, *E. coli* HB101 and LE392, together with the same strains transformed with the cloning vector pHC79, as well as the plasmid (pPL376) into which the carotenoid biosynthetic genes had been cloned, were used.

When HB101 and its plasmid-containing derivatives were treated with far-UV (200–290 nm), the inactivation kinetics were indistinguishable.[16] This result is exactly what might have been expected since the inactivating events produced by far-UV are based on the direct absorption of these wavelengths by DNA leading to the formation of pyrimidine dimers among other minor lesions. Furthermore, since carotenoids are associated with the cell membrane,[1] carotenoids would not be expected to offer protection from far-UV inactivation. These results are consistent with those reported with *N. crassa* in which the inactivation kinetics of wild-type (carotenoid containing) and albino (carotenoidless) conidia following

[19] R. W. Tuveson and R. B. Jonas, *Photochem. Photobiol.* **30**, 667 (1979).

treatment with far-UV were essentially identical.[9] Using the same *E. coli* strains described here, Perry *et al.*[7] reported that the pigmented and nonpigmented strains were equally sensitive to inactivation by UV Since these investigators did not specify the wavelengths investigated, we assume that they were using far-UV, explaining the absence of carotenoid protection.

When *E. coli* strains HB101 and HB101(pHC79) were treated with broad-spectrum near-UV, the inactivation kinetics were essentially identical. The results with the carotenoid-producing strain [HB101(pPL376)] were different, showing apparent protection by carotenoids at higher fluence levels.[16] Apparently carotenoids do offer some protection against the inactivating effects of near-UV, at least at higher fluences. The complex character of the inactivation curve obtained with the carotenoid-producing strain [HB101(pPL376)] reflects the possibility that both DNA and the membrane are important lethal targets for near-UV, as has been suggested.[20,21] These observations are consistent with results obtained using an *N. crassa* carotenoid-producing and a carotenoidless mutant, showing that carotenoids offer only limited protection against the inactivating effects of near-UV alone.[9,10] Perhaps the fact that both *E. coli* and *N. crassa* carotenoids offer limited protection against near-UV inactivation means that carotenoids are capable of quenching only $^1O_2$ or triplet state photosensitizers. Near-UV inactivation is oxygen dependent and apparently generates chiefly superoxide, leading to H_2O_2 and ultimately hydroxyl radical.[22,23] It cannot be excluded that near-UV may also generate a limited amount of $^1O_2$, thus accounting for the limited protection seen when carotenoid-producing cells are treated with near-UV alone. If carotenoids can protect by quenching hydroxyl radical, then it should be possible to demonstrate that the carotenoid-producing strains [HB101(pPL376) or LE392(pPL376)] are protected against inactivation by H_2O_2, which in the presence of Fe(II) leads to hydroxy radical. Using the glucose–glucose oxidase H_2O_2-generating system,[23] we have attempted to demonstrate such protection with the *E. coli* strains described here with results that are uniformly negative. We conclude that under the conditions of this assay, carotenoids do not protect against H_2O_2 and ultimately hydroxyl radical.

The *E. coli* carotenoidless [HB101, HB101(pHC79)] and carotenoid-producing strains were tested with a variety of photosensitizing molecules (phototoxins) activated by near-UV. It is important to note that the maximum near-UV fluence used to activate the phototoxins described below

[20] S. H. Moss and K. C. Smith, *Photochem. Photobiol.* **33,** 202 (1981).
[21] A. Eisenstark, *Environ. Mol. Mutagen.* **10,** 317 (1987).
[22] L. J. Sammartano and R. W. Tuveson, *Photochem. Photobiol.* **40,** 607 (1984).
[23] L. J. Sammartano, R. W. Tuveson, and R. Davenport, *J. Bacteriol.* **168,** 13 (1986).

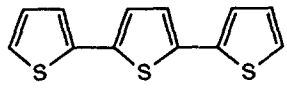

FIG. 1. Chemical structure of α-terthienyl.

results in no inactivation of the three *E. coli* strains when used alone. Psoralen and 8-methoxypsoralen are near-UV-activated molecules that inactivate chiefly by forming cycloadducts to DNA. Since the inactivation by these molecules is not oxygen dependent, it was expected that protection by carotenoids would not occur. This is exactly what was observed.[16]

When the carotenoidless and carotenoid-producing strains were treated with near-UV-activated molecules that exhibited oxygen dependence, the degree of protection offered by carotenoids depended on the particular molecule. For example, carotenoids offered only modest protection against inactivation by near-UV-activated phenylheptatriyne, suggesting that this oxygen-dependent molecule probably generated chiefly superoxide leading to H_2O_2 and, ultimately, hydroxyl radical *in vivo*. However, the fact that some protection is afforded by the presence of carotenoids must mean that some singlet oxygen is also formed *in vivo*. Of all the near-UV-activated oxygen-dependent molecules investigated to date, α-terthienyl (α-T, Fig. 1) represents one in which the available evidence suggests that the chief damaging product is $^1O_2$.[24] The target for attack by α-T plus near-UV seems to be the cell membrane since carotenoids do strongly protect against inactivation by this near-UV-activated molecule and it appears to be incapable of inducing mutations. Because the α-T molecule is extremely hydrophobic, it may simply be incapable of entering cells to attack DNA. This possibility was checked by using purified *Haemophilus influenzae* transforming DNA and pBR322 DNA.[25] Using these DNAs it was shown that near-UV-activated α-T did not destroy *H. influenzae* transforming activity. However, such treatment was capable of converting supercoiled pBR322 DNA to the open circular form. Open circular pBR322 DNA is capable of transforming *E. coli* probably because the cell contains sufficient ligase to repair nicks in the DNA backbone. The fact that *H. influenzae* DNA seemed not to be affected by treatment with α-T plus near-UV may simply reflect the fact that the cell repairs any single-strand DNA nicks during the process of uptake. If α-T does penetrate cells and produces single-strand breaks following treatment with near-UV, such nicks are probably rapidly repaired by ligase and would not influence survival.

[24] K. R. Downum, R. W. E. Hancock, and G. H. N. Towers, *Photochem. Photobiol.* **36,** 517 (1822).

[25] T. P. Wang, J. Kagan, R. W. Tuveson, and G. R. Wang, *Photochem. Photobiol.* **53,** 463 (1991).

The effects of α-T plus near-UV have been reinvestigated using recipient *E. coli* strains HB101 and LE392 carrying plasmids pHC79 (original cloning vector) and pPL376 (carrying genes specifying carotenoid biosynthesis) as well as plasmid pdel 16 (a deletion plasmid resulting in the accumulation of lycopene). The results of these experiments (Fig. 2) confirm our previous results with *E. coli* HB101[16] and LE392[13] and their plasmid-carrying derivatives. As expected, the carotenoid-producing strains (carrying plasmid pPL376) are protected against inactivation by α-T plus near-UV. The strain carrying the plasmid allowing for the accumulation of lycopene (pdel16) is as sensitive to inactivation by α-T plus near-UV as is the recipient strain or the strain carrying the cloning vector (pHC79). This result supports the view that acyclic carotenes probably do not function to quench triplet state photosensitizers or $^1O_2$. The only

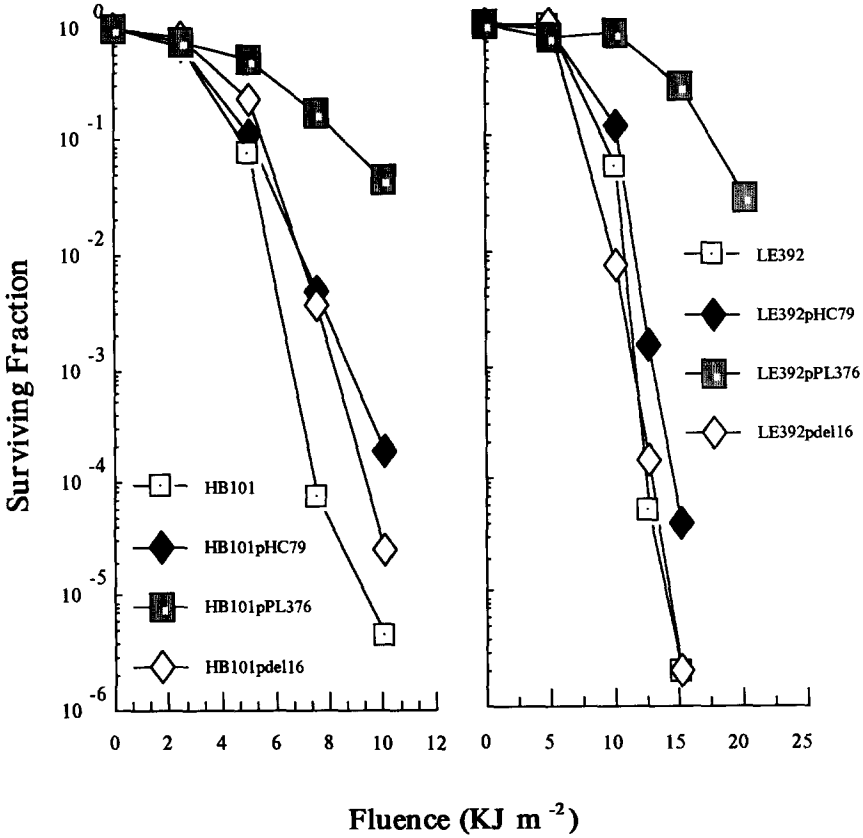

FIG. 2. Near-UV fluence–response curves for various strains of *Escherichia coli* in the presence of α-terthienyl (10 μg/ml).

difference between the experiments involving HB101 and its derivatives and LE392 and its derivatives is that the HB101 strains are about twice as sensitive to inactivation by α-T plus near-UV when compared to the LE392 strains (compare the fluence axes for the two sets of experiments).

Conclusions

The *E. coli* strains described here can be used to determine whether carotenoids offer protection against inactivation by a particular photosensitizer, suggesting that either (1) the triplet state of the photosensitizer is quenched or (2) $^1O_2$ is quenched in the case of oxygen-dependent photosensitizers. Protection by carotenoids also suggests that the membrane is an important target for lethal events. The absence of protection by carotenoids against inactivation by a photosensitizer can result from the fact that the photosensitizer either (1) targets sites other than the membrane for lethal damage or (2) generates superoxide leading to H_2O_2 and, ultimately, hydroxyl radical in the case of oxygen-dependent photosensitizers. It is probable in the case of most oxygen-dependent photosensitizers that the partitioning of the sensitizer into the aqueous and lipid portion of the cell determines whether it generates chiefly $^1O_2$ or superoxide and, ultimately, hydroxy radical (electron transfer reaction).

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[29] Biosynthesis of Carotenoids: An Overview

By T. W. GOODWIN

Introduction

It was in the early 1930s that the three basic carotenoid structures lycopene (I), α-carotene (II), and β-carotene (III) were elucidated.[1] All known naturally occurring carotenoids (about 400) are variations on these

[1] O. Isler, ed., "Carotenoids." Birkhauser, Basel, 1971.