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Variation in Expression of Carotenoid Genes in Transformed *E. coli* Strains

Eleanore T. Wurtzel*, Gregorio Valdez, and Paul D. Matthews

Department of Biological Sciences, Lehman College and The Graduate Center, The City University of New York, 250 Bedford Park Boulevard West, Bronx, New York 10468 USA

running title: Carotenoids in *E. coli* Strains

*Corresponding author:
Dr. Eleanore Wurtzel
Phone: 718-960-8643
Fax: 718-960-7267 or 718-960-8236
E-mail: etwlc@cunyvm.cuny.edu

Abstract

The carotenoid biosynthetic gene cluster of *Erwinia uredovora* has been used to condition the expression of colored carotenoids in various heterologous systems including *Escherichia coli*. Heterologous expression of these genes in *E. coli* is a powerful tool for investigating carotenoid gene function and for screening for new carotenoid related genes. We have assessed fourteen *E. coli* strains for level and stability of expression of *E. uredovora* carotenoid genes. Lycopene and zeaxanthin producing strains were examined for colony pigmentation and for accumulated carotenoids extractable from liquid cultures. Strains varied independently for colony pigmentation and stability of expression. The strain differences observed did not appear to be due to the particular carotenoid accumulated. The strains TOP10 F' and AB2463 exhibited the best accumulation of carotenoids. Recommendations are made for heterologous complementation expression screening for higher plant cDNA libraries and for improvements to this system.

Introduction

Carotenoids, a large class of yellow to orange hydrophobic pigments, photoprotect nonphotosynthetic microorganisms in high light aerobic environments. In photosynthetic organisms, carotenoids have additional functions as accessory pigments in photosynthesis. A property unique to plants, certain carotenoids are hormone precursors (2).

In microorganisms, such as *Erwinia uredovora* or *Erwinia herbicola*, the carotenoid biosynthetic pathway is specified by a gene cluster encoding biosynthetic enzymes that function in a pathway beginning with the synthesis of geranylgeranyl pyrophosphate (GGPP) and ending in the synthesis of zeaxanthin glucosides (9, 10, 20, 29). All or part of the *E. uredovora* and *E. herbicola* carotenoid gene clusters have been introduced into *Escherichia coli*, conferring upon this otherwise nonpigmented bacterium, the ability to accumulate a range of colored carotenoids (20 , 29). Since carotenoids are derived from isoprenoid precursors (5, 27), *E. coli* can accumulate carotenoids by coupling an endogenous isoprenoid biosynthetic pathway with enzymes encoded by transformed genes of carotenogenic organisms such as *E. uredovora*. In fact, the biosynthetic pathway can be reconstituted *in vivo* even if the enzymes are of such diverse origin as those encoded by bacteria and plants (for examples, see (4, 15, 21)).

The expression of carotenoid genes in *E. coli* has been useful for identifying function of gene products (4, 6, 8, 14-17, 19, 23, 26), dissection of the pathway (11, 28), study of transcriptional regulators of carotenogenic genes (24), and isolation of new genes encoding enzymes of the carotenoid biosynthetic pathway (13, 18) or enzymes catalyzing the synthesis of carotenoid precursors (22).

The huge diversity and distribution of natural carotenoids present a challenge for isolation and comparative understanding of a large array of genes and gene products. The utility of *E. coli* for study of carotenoid genes, depends on adequate expression of those genes giving visible changes in *E. coli* pigmentation that can also be detected by analytical methods such as spectrophotometry or HPLC (high pressure liquid chromatography). Yet, expression of carotenoid genes is not consistent among *E. coli* strains (unpublished observations). Therefore, we compared fourteen *E. coli* strains for level and stability of expression of genes encoding carotenoid biosynthetic enzymes. The strains analyzed included those with genetic markers known to minimize recombination of cloning vectors or to promote infection by phage cloning vectors. *E. coli* strains were transformed with one of two

plasmids encoding portions of the *E. uredovora* biosynthetic pathway, resulting in accumulation of either lycopene or zeaxanthin. We observed that the *E. coli* strains tested varied widely in carotenoid accumulation and stability of expression.

Materials and Methods

E. coli K-12 strains (Table 1) were transformed by the $CaCl_2$ protocol (25) with plasmids pACCART-EIB and pACCAR25ΔcrtX carrying gene clusters from *Erwinia uredovora* for biosynthesis of lycopene and zeaxanthin, respectively (20). Transformants selected and maintained by resistance to 170 µg/mL chloramphenicol were confirmed by visual assessment of colony color on two day old Luria-Bertani (LB) plates and by plasmid DNA extraction from liquid culture (25). For quantitative extraction of carotenoids, a 10 µl stationary culture from a frozen glycerol stock was inoculated into 10 mL LB with antibiotic, grown overnight at 37 degree Celsius to stationary phase and 10 µl inoculated into 100 ml LB with antibiotic in a 250 ml Erlenmeyer flask, incubated for 24 hrs at 37 degree Celsius with agitation of *c.a*. 150 rpm. Aliquots were used to determine cell density and total protein concentration (Lowry Protein Determination Kit, Sigma Chemical Co., St. Louis, MO). Wet weight of cell pellets from 40 mL cultures were determined before carotenoid extraction. Lycopene was extracted according to Linden (16); zeaxanthin was extracted with modifications of Linden's protocol for improved yields. In reduced light, a 40 ml culture was pelleted by centrifugation, drained and resuspended in 10 ml methanol with 6% KOH. Ten ml saturated NaCl was added, and zeaxanthin partitioned into 7.5 ml petroleum ether. The partitioning was repeated with an additional 7.5 ml petroleum ether, and the combined extracts dried at room temperature in a Savant Speedvac centrifugal evaporator. Carotenoid concentrations were determined spectrophotometrically with molar extinction coefficients of 184900 for lycopene in petroleum ether at 470 nm absorbance and 133400 for zeaxanthin in petroleum ether at 449 nm absorbance (3). Values for extractable carotenoids showed high correlation with values normalized to protein concentration or to cell titer (data not shown).

Results

Fourteen *E. coli* strains were chosen from various sources, including those commonly used for gene transformation. These strains were transformed with one of two plasmids that differed in the number of *E. uredovora* genes encoding carotenoid biosynthetic enzymes. Lycopene accumulated in

transformants containing pACCART-EIB, which has only part of the gene cluster, *crtE*, *crtB*, and *crtI*, encoding GGPP synthase, phytoene synthase, and phytoene desaturase, respectively. Zeaxanthin accumulated in transformants containing the second plasmid, pACCAR25ΔcrtX, which encodes the entire gene cluster except *crtX* (20).

As shown in Table 1, the fourteen strains were ranked based on carotenoid pigmentation of transformants grown on a solid medium. We observed that transformed strains varied widely for both level of pigmentation and for stability of expression. When grown on plates, TOP10 F' and AB2463 showed the highest accumulation of pigments, whereas SURE and Y1088 showed the lowest accumulation. Except strain V73, the accumulation of lycopene and zeaxanthin did not differ for a particular strain. Plating density and colony size affected pigmentation markedly: small (<1mm diameter) colonies, resulting from high density (>12 colonies/ cm^2) plating, showed little pigmentation, regardless of plasmid present. Pigment accumulation could be enhanced by growth over several days, although differences among strains remained. At lower plating densities, pigment accumulation was improved.

In some strains, such as SK2267, V71, KL168, and SK3451, expression was unstable: colonies showed sectoring of pigmentation, and many unpigmented colonies were present among chloramphenicol resistant primary transformants.

Strains grown in liquid culture also reflected variation in accumulation, although the range was not as dramatic as when strains were grown on solid medium. When TOP10 F' and SURE cells were grown in liquid culture, the TOP10 F' culture accumulated almost twice as much lycopene (approximately 100 µg/gm wet cell weight) as compared to SURE cells.

Discussion

Carotenoid pigmentation in fourteen strains grown on solid medium varied widely. There was also no obvious correlation between genetic markers and pigmentation (Table 1). It is likely that unidentified markers affect pigment accumulation. For example, since the pathway utilizes isoprenoid precursors, expression of loci controlling isoprenoid biosynthesis or pathways competing for precursors pools (5), might affect overall carotenoid accumulation from strain to strain. Examination of isoprenoid levels in these strains might reveal differences in isoprenoid pools that would support the observed differences in pigment accumulation. Alternatively, strains may vary in level of expression of the heterologous *E. uredovora* genes that could also account for differences in

carotenoid accumulation between strains.

The carotenoid pigments themselves, which are hydrophobic and may perturb the membrane through association, could also affect accumulation. But as shown in Table 1, in most cases, the accumulation and stability of expression was independent of carotenoid accumulated.

Instability in expression, such as sectored colonies and colony to colony pigment variation, was also observed. However, this instability appeared to be independent of pigmentation level. For example, both HB101 and KL168 showed medium pigmentation, although expression in KL168 was very unstable compared with HB101. Similarly, strains showing weak pigmentation, such as SURE and Y1088, were not necessarily unstable. Perhaps, these strains vary in stability of the plasmid. Both plasmids have in common *crtE*, *crtB*, and *crtI*, encoding GGPP synthase, phytoene synthase, and phytoene desaturase, respectively, but pACCAR25ΔcrtX additionally contains *crtX*, *crtY* and *crtZ* encoding zeaxanthin glucosyl transferase, lycopene cyclase, and β-carotene hydroxylase, respectively, with a frameshift mutation in *crtX*. The gene cluster of pACCART-EIB is 3.8 kb, while the cluster of pACCAR25ΔcrtX is 6.5 kb. Since some strains showing instability of carotenoid pigmentation were recombination deficient (for example, *recA*), it is unlikely that the observed instability was a result of marker loss from the plasmid.

We have identified several strains suitable for studies of carotenoid biosynthesis, such as functional analysis of cloned genes. Although *E. coli* strains have been used for complementation screening using M13-based cDNA expression libraries, we found that some stable carotenoid accumulating strains became unstable when superinfected with such M13-based cDNA expression libraries (data not shown). Therefore, some caution is needed in using even the stable and strongly pigmented strains for such studies. Since we observed that optimal colony pigmentation is obtained with low density plating, screening for higher plant genes encoding carotenoid enzymes will be more difficult as these genes are generally expressed at low levels (for example, see (15)). To screen one million clones by complementation, would require one hundred and forty 625cm^2 screening plates. Methods for selection of carotenoid producing cells in liquid culture could greatly improve the efficacy of screening. For example, fluorometric flow cytometric cell sorting devices have been used to isolate carotenoid producing yeasts (1). Such devices might prove useful for screening by complementation where gene expression alters carotenoid pigmentation compared to background cells. Advantage could be also be taken of the photoprotective (30) or singlet-oxygen toxicity-protective properties (7, 12) of carotenoids to select against non-pigmented cells before plating.

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References

1. **An, G. H., J. Bielich, R. Auerbach, and E. A. Johnson.** 1991. Isolation and characterization of carotenoid hyperproducing mutants of yeast by flow cytometry and cell sorting. Biotechnology (N Y). **9**(1)**:**70-3.
2. **Bartley, G. E., and P. A. Scolnik.** 1995. Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell. **7**(7)**:**1027-1038.
3. **Britton, G. (ed.).** 1975. Carotenoids, vol. 1B. Birkhauser, Berlin.
4. **Chamovitz, D., N. Misawa, G. Sandmann, and J. Hirschberg.** 1992. Molecular cloning and expression in *Escherichia coli* of a cyanobacterial gene coding for phytoene synthase, a carotenoid biosynthesis enzyme. FEBS Lett. **296**(3)**:**305-10.
5. **Chappell, J.** 1995. Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Ann Rev Plant Physiol Plant Mol Biol. **46:**521-547.
6. **Cunningham, F. X., Jr., D. Chamovitz, N. Misawa, E. Gantt, and J. Hirschberg.** 1993. Cloning and functional expression in *Escherichia coli* of a cyanobacterial gene for lycopene cyclase, the enzyme that catalyzes the biosynthesis of β-carotene. FEBS Lett. **328**(1-2)**:**130-8.
7. **Dahl, T. A., W. R. Midden, and P. E. Hartman.** 1989. Comparison of killing of gram-negative and gram-positive bacteria by pure singlet oxygen. J Bacteriol. **171**(4)**:**2188-94.
8. **Hugueney, P., S. Romer, M. Kuntz, and B. Camara.** 1992. Characterization and molecular cloning of a flavoprotein catalyzing the synthesis of phytofluene and ζ-carotene in Capsicum chromoplasts. Eur J Biochem. **209**(1)**:**399-407.
9. **Hundle, B., M. Alberti, V. Nievelstein, P. Beyer, H. Kleinig, G. A. Armstrong, D. H. Burke, and J. E. Hearst.** 1994. Functional assignment of *Erwinia herbicola* Eho10 carotenoid genes expressed in *Escherichia coli*. Mol Gen Genet. **245**(4)**:**406-416.
10. **Hundle, B. S., P. Beyer, H. Kleinig, G. Englert, and J. E. Hearst.** 1991. Carotenoids of *Erwinia herbicola* and an *Escherichia coli* HB101 strain carrying the *Erwinia herbicola* carotenoid gene cluster. Photochem Photobiol. **54**(1)**:**89-93.
11. **Hundle, B. S., O. B. DA, P. Beyer, H. Kleinig, and J. E. Hearst.** 1993. *In vitro* expression and activity of lycopene cyclase and β-carotene hydroxylase from *Erwinia herbicola*. FEBS Lett. **315**(3)**:**329-34.
12. **Kagan, J., T. P. Wang, I. A. Kagan, R. W. Tuveson, G. R. Wang, and J. Lam.** 1992. Photosensitization by 2-chloro-3,11-tridecadiene-5,7,9-triyn-1-ol: damage to erythrocyte

membranes, *Escherichia coli*, and DNA. Photochem Photobiol. **55**(1)**:**63-73.

13. **Kajiwara, S., T. Kakizono, T. Saito, K. Kondo, T. Ohtani, N. Nishio, S. Nagai, and N. Misawa.** 1995. Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis from *Haematococcus pluvialis*, and astaxanthin synthesis in *Escherichia coli*. Plant Mol Biol. **29**(2)**:**343-352.
14. **Kuntz, M., S. Romer, C. Suire, P. Hugueney, J. H. Weil, R. Schantz, and B. Camara.** 1992. Identification of a cDNA for the plastid-located geranylgeranyl pyrophosphate synthase from *Capsicum annum*: correlative increase in enzyme activity and transcript level during fruit ripening. Plant J. **2**(1)**:**25-34.
15. **Li, Z.-h., P. D. Matthews, B. Burr, and E. T. Wurtzel.** 1996. Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway. Plant Molecular Biology. **30:**269-279.
16. **Linden, H.** 1991. Functional complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenoids. Z. Naturforsch. **46**(c)**:**1045-1051.
17. **Linden, H., N. Misawa, T. Saito, and G. Sandmann.** 1994. A novel carotenoid biosynthesis gene coding for ζ-carotene desaturase: functional expression, sequence and phylogenetic origin. Plant Mol Biol. **24**(2)**:**369-79.
18. **Lotan, T., and J. Hirschberg.** 1995. Cloning and expression in *Escherichia coli* of the gene encoding β-C- 4-oxygenase, that converts β-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. FEBS Lett. **364**(2)**:**125-128.
19. **Math, S. K., J. E. Hearst, and C. D. Poulter.** 1992. The *crtE* gene in *Erwinia herbicola* encodes geranylgeranyl diphosphate synthase. Proc Natl Acad Sci U S A. **89**(15)**:**6761-4.
20. **Misawa, N., M. Nakagawa, K. Kobayashi, S. Yamano, Y. Izawa, K. Nakamura, and K. Harashima.** 1990. Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. J Bacteriol. **172**(12)**:**6704-12.
21. **Misawa, N., M. R. Truesdale, G. Sandmann, P. D. Fraser, C. Bird, W. Schuch, and P. M. Bramley.** 1994. Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation *in vivo* and *in vitro*, and functional analysis of the various truncated gene products. J Biochem (Tokyo). **116**(5)**:**980-985.
22. **Ohnuma, S., M. Suzuki, and T. Nishino.** 1994. Archaebacterial ether-linked lipid

biosynthetic gene. Expression cloning, sequencing, and characterization of geranylgeranyl-diphosphate synthase. J Biol Chem. **269**(20)**:**14792-7.

23. **Pecker, I., D. Chamovitz, H. Linden, G. Sandmann, and J. Hirschberg.** 1992. A single polypeptide catalyzing the conversion of phytoene to ζ-carotene is transcriptionally regulated during tomato fruit ripening. Proc Natl Acad Sci U S A. **89**(11)**:**4962-6.
24. **Penfold, R. J., and J. M. Pemberton.** 1994. Sequencing, chromosomal inactivation, and functional expression in *Escherichia coli* of *ppsR*, a gene which represses carotenoid and bacteriochlorophyll synthesis in *Rhodobacter sphaeroides*. J Bacteriol. **176**(10)**:**2869-76.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
26. **Sandmann, G.** 1993. Carotenoid analysis in mutants from *Escherichia coli* transformed with carotenogenic gene cluster and *Scenedesmus obliquus* mutant C-6D. Methods Enzymol. **214:**341-7.
27. **Sandmann, G.** 1994. Carotenoid biosynthesis in microorganisms and plants. Eur J Biochem. **223**(1)**:**7-24.
28. **Sandmann, G., and N. Misawa.** 1992. New functional assignment of the carotenogenic genes *crtB* and *crtE* with constructs of these genes from Erwinia species. FEMS Microbiol Lett. **69**(3)**:**253-7.
29. **Sandmann, G., W. S. Woods, and R. W. Tuveson.** 1990. Identification of carotenoids in *Erwinia herbicola* and in a transformed *Escherichia coli* strain. FEMS Microbiol Lett. **59**(1-2)**:**77-82.
30. **Tuveson, R. W., R. A. Larson, and J. Kagan.** 1988. Role of cloned carotenoid genes expressed in *Escherichia coli* in protecting against inactivation by near-UV light and specific phototoxic molecules. J Bacteriol. **170**(10)**:**4675-80.

TABLE 1. Descending rank of *E. coli* strains transformed with *E. uredovora* carotenoid genes.

Strains Ranked for Carotenoid Pigmentation

	STRAIN	SOURCE	GENOTYPIC MARKERS	RATING[a]/comment[b]
1	TOP10 F'	Invitrogen	F'{tet^r} *mcrA* Δ(*mrr hsdRMS mcrBC)* ϕ80Δ*lac*-Δ*M15* Δ*lac*X*74 deoR recA1 araD139* Δ*(ara, leu)7697 galU galK* λ$^-$ *rpsL endA1 nupG*	high
2	AB2463	CGSC#2463	F$^-$ *thr-1 ara-14 leuB6* Δ*(gpt-proA)62 lacY1 tsx-33 qsr'-0 glnV44(AS) galK2* λ$^-$ *rac-0 hisG4(Oc) rfbD1 recA13 rpsL31(strR) kdgK51 xylA5 mtl-1 argE3(Oc) thi-1*	high
3	HB101	B R L	F$^-$ *mcrB mrr hsdS20(r_B^-,m_B^-) recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20(Str^r) xy15* λ$^-$ *leu mtl1*	medium
4	SOLR	Stratagene	[F' *proAB lac1^q Z*Δ *M15*] *e14-(mcrA)* Δ*(mcrCB-hsdSMR-mrr)171 sbcC recB recJ uvrC umuC::*Tn5(kan^r) *lac gyrA96 relA1 thi-1 endA1* λ^R Su$^-$ (non-suppressing)	medium
5	V73	CGSC#6736	F$^-$ *tsx-33 galK2* λ$^-$ *rac-0 hisG4(Oc) recC73 recC1001(χ) argA21 rpsL31(strR) kdgK51 xylA5 mtl-1 recF143 bglR17 Met-(ts)*	medium (lyc), high (zea)
6	AB1884	CGSC#1884	F$^-$ *thr-1 ara-14 leuB6* Δ*(gpt-proA)62 lacY1 tsx-33 qsr'-0 glnV44(AS) galK2* λ$^-$ *rac-0 uvrC34 hisG4(Oc) rfbD1 mgl-51 rpsL31(strR) kdgK51 xylA5 mtl-1 argE3(Oc) thi-1*	medium
7	AB2480	CGSC#2480	Δ(*gpt-pro*A)*62 lacY1 tsx-33 glnV44(AS) galK2* λ$^-$ *recA13 rpsL31(strR)* or *rpsL8 xylA5 mtl-1 thi-1 uvrA6*	medium
8	AB1886	CGSC#1886	F$^-$ *thr-1 ara-14 leuB6* Δ*(gpt-proA)62 lacY1 tsx-33 qsr'-0 glnV44(AS) galK2* λ$^-$ *rac-0 hisG4(Oc) rfbD1 mgl-51 rpsL31(strR) kdgK51 xylA5 mtl-1 argE3(Oc) thi-1 uvrA6*	medium
9	SK2267	CGSC#12959	F$^-$ *lac*Z4 or *lac*-61 *gln*V44(AS) *gal*-44 λ$^-$ *sbc*B15 *rec*A1 *end*A1 *rfa*-([C]) *thi*-1 *hsd*R4 *ton*-58	medium /unstable
10	V71	CGSC#6734	F$^-$ *tsx-33 galK2* λ$^-$ *rac-0 hisG4(Oc) recC73 recC1003(χ) argA21 rpsL31(strR) kdgK51 xylA5 mtl-1 recF143 bglR17 Met-(ts)*	medium /unstable
11	KL168	CGSC#4202	Hfr λ$^-$ *e14- relA1 recB21 thi-1 deoB13*	medium/very unstable
12	SK3451	CGSC#6882	F$^-$Δ(*lac*Y-*lac*Z)286 *tsx*-3 *gln*V44(AS) λ$^-$ *lac*Z95=ϕ80dII*lac*Z9 *his*G4(Oc) *rps*L281(strR) *xyl*A7 *mtl*-1 *met*E46 *uvrD282*::Tn5 *argH1*(del)	medium/very unstable
13	Y1088	Stratagene	*sup*E *sup*F *met*B *trp*R *hsd*R$^-$ *hsd*M$^+$ *ton*A21 *str*A$^-$ *lac*U169 *pro*C::Tn5 (pMC9 *lac*I)	low
14	SURE	Stratagene	[F' *proAB lacI^{q}Z*Δ*M15* Tn10 (tet^r)] *e14-(mcrA)* Δ*(mcrCB-hsdSMR-mrr)171 sbcC recB recJ umuC::*Tn5(kan^r) *uvrC supE44 lac gyrA96 relA1 thi-1 endA1*	low

[a] High, medium, and low are relative ratings of carotenoid accumulation based on visual assessment of strain pigmentation after 48 hrs of growth on a solid medium. Unless indicated otherwise, the rating is identical for lycopene (lyc)-expressing cells and zeaxanthin (zea)-expressing cells.

[b] unstable, many colonies sectored for pigmentation and marked pigment variation between colonies; very unstable, a majority of unpigmented colonies.