CAROTENOIDs OF Erwinia herbicola AND AN Escherichia coli HB101 STRAIN CARRYING THE Erwinia herbicola CAROTENOID GENE CLUSTER

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Abstract—Carotenoid pigments of Erwinia herbicola and a transformed strain of Escherichia coli carrying the carotenoid biosynthesis gene cluster of E. herbicola have been analyzed. Both organisms are capable of making essentially the same carotenoids, indicating that all of the genes required for the biosynthesis of the wild type E. herbicola carotenoids have been transformed intact into E. coli. The major products in both species of bacteria are β-cryptoxanthin glucoside, zeaxanthin monoglucoside and zeaxanthin diglucoside. These compounds are the first example of secondary, non-allylic carotenoid glucosides. The absolute configuration 3R,3'R for zeaxanthin diglucoside was determined from its circular dichroism spectrum. Both species of bacteria also accumulate small amounts of hydrocarbon carotenes with similar cis/trans isomerization states.

INTRODUCTION

Erwinia herbicola is a gram negative non-photosynthetic bacterium which belongs to the enterobacteria. Certain strains of this bacterium are also capable of providing nucleation sites for ice crystal formation on plant tissue, leading to an increased susceptibility to frost damage to crops (Orser et al., 1985). Many epiphytic strains of E. herbicola found in aerial plant parts produce yellow pigments (Mergaert et al., 1984). These pigments were initially suggested by Starr (1981) to be cyclic glycosylated carotenoids. The genes for carotenoid synthesis in E. herbicola are coded by a chromosomal fragment. Perry et al. (1986) constructed a genomic library of E. herbicola EholO by inserting partially Sau3A digested DNA into the BamH1 site of cosmid pHC79. Escherichia coli HB101 was used as a host strain. From this library it was demonstrated that the genes for yellow pigment production lie within a 12.4 kb sequence (Perry et al., 1986). The plasmid constructed by Perry et al. is illustrated in Fig. 1. DNA sequence analysis in our laboratory concluded 12 possible open reading frames (Orf's)† (Fig. 1). In order to assign biochemical function to these Orf's, it is essential to establish the carotenoid biosynthesis pathway in both E. herbicola and transformed E. coli. Initial characterization of the yellow pigments produced in these E. coli HB101(pPL376)

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†Abbreviations: CD, circular dichroism; GGPP, geranylgeranyl pyrophosphate; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; Orf's, open reading frames; PPPP, prephytoene pyrophosphate.
HB101(pPL376). We also suggest a possible carotenoid biosynthetic pathway in these organisms.

MATERIALS AND METHODS

Organisms and growth conditions. The E. herbicola Eho10 strain (ATCC 39368) was grown in Luria-Bertani (LB) broth containing 10 g of tryptone (Difco Laboratories, Detroit, MI), 5 g of yeast extract (Difco), and 10 g of NaCl/L (Miller, 1972) at pH 7.6, in shaking cultures at 28°C. Large batches of cells (18 L) were grown to stationary phase by constantly bubbling air through the culture while gently magnetically stirring. The E. herbicola carotenoid gene cluster carried on plasmid pPL376 (Perry et al., 1986) was maintained in E. coli HB101 grown at 37°C by selection for ampicillin resistance using 100 μg/mL ampicillin (Sigma Chemical Co., St. Louis, MO). Cells for carotenoid analysis were grown in 0.5 L LC broth containing 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), and 5 g of NaCl/L (Marrs, 1981), containing 100 μg/mL ampicillin by shaking cultures at 37°C.

Extraction and separation of carotenoids. For determining crude carotenoid content lyophilized E. herbicola and E. coli HB101(pPL376) cells (10 mg each) were extracted (2 x 1 mL) with chloroform/methanol (2:1, vol/vol), because the classical solvent acetone did not quantitatively extract the most polar pigment. Carotenoid content was calculated based on A_{478}=2000 at λ_{max} (Nybraaten and Liaaen-Jensen, 1974). For separation of individual components the extract was dried with a stream of nitrogen or in a rotary evaporator and dissolved in a mixture of pyridine/acetic anhydride (1:1, vol/vol) with catalytic amounts of 4-(dimethylamino)pyridine for quantitative acetylation. After a 30 min incubation methanol was added to destroy the remaining acetic anhydride. The carotenoid peracetates were transferred into petroleum benzene/ether (1:1, vol/vol) in a separatory funnel, and the polar solvents were removed by repeated washings with water. The carotenes and carotenoid peracetates were separated on silica gel thin layer plates using the solvent mixture, petroleum benzene/diethyl ether/acetone (40:10:5, vol/vol). As revealed by a fluorescence reagent (Kleinig and Lempert, 1989), the pigment bands were essentially free of contaminating phospho- or glycolipids. Pigment bands were scraped off, eluted with acetone and subjected to a further separation on HPLC. A Nucleosil 5 μm C_{18} reversed phase column was used with either acetonitrile or 3% water in acetonitrile as the eluent. The carotene fraction was dried with a stream of nitrogen and dissolved in a mixture of benzene/ether (1:1, vol/vol) in a separatory funnel. The carotenes and carotenoid peracetates were transferred into petroleum benzene/ether (1:1, vol/vol) in a separatory funnel, and the polar solvents were removed by repeated washings with water. The carotenes and carotenoid peracetates were separated on silica gel thin layer plates using the solvent mixture, petroleum benzene/diethyl ether/acetone (40:10:5, vol/vol). As revealed by a fluorescence reagent (Kleinig and Lempert, 1989), the pigment bands were essentially free of contaminating phospho- or glycolipids. Pigment bands were scraped off, eluted with acetone and subjected to a further separation on HPLC. A Nucleosil 5 μm C_{18} reversed phase column was used with either acetonitrile or 3% water in acetonitrile as the eluent. The flow rate was 1.3 mL/min. This separation system was used for the peracetates as well as for the carotene fraction (Beyer et al., 1989).

Mass and nuclear magnetic resonance spectroscopy. 1H-NMR spectra were recorded at 400 MHz in CDCl_{3} (99.98% D, CEA) with tetramethylsilane TMS as internal standard on a Brucker AM-400 FT-NMR spectrometer with an ASPECT 3000 computer. Low resolution mass spectra were measured on a MAT 90 spectrometer from Finnigan-MAT (Bremen, FRG) using electron impact ionization at 70 eV. The m/z of relevant peaks and their relative intensities, in parentheses, are given.

The CD spectrum was measured on a Jobin-Yvon CD 6 instrument (France) with ethanol as the solvent.

RESULTS

Both bacterial strains, E. herbicola and E. coli HB101(pPL376) contained trace amounts of carotenes and four main polar pigments. The polar pigments of the two strains showed identical absorption spectra (maxima at 478, 452, shoulder at 430 nm; in acetone), which is identical with that of β-carotene or zeaxanthin. Acetylation analysis revealed the presence of several hydroxy groups, pointing to three glycosylated xanthophylls (pigment 1–pigment 3, see Fig. 2). Furthermore, zeaxanthin was identified in E. herbicola. The carotenones were identified by HPLC analysis and by their absorption spectra (Tables 1 and 2).

In the hydrocarbon carotene fractions two or more cis/trans-isomers of each compound were detected; the isomer pattern was the same in both strains. The glycosides occurred mainly in the all-
Carotenoids of *Erwinia herbicola*

Figure 3. Proposed carotenoid biosynthesis pathway in *E. herbicola*. Dehydrogenation intermediates not shown between phytoene, the first C_{50} carotenoid, and lycopene are phytofluene, \( \epsilon \)-Carotene, and neurosporene, respectively (see Beyer et al. 1989 for structures). The biosynthesis of the carotenoid glucosides from lycopene requires a minimum of three types of reactions: ring cyclization, hydroxylation, and glucosylation. Numbers in parentheses correspond to the acetylated derivative described in the text.
trans configuration accompanied by several cis-isomers, two of which exhibited a main absorption peak at 445 nm and a prominent cis-peak at 340 nm. This again was observed identically in both strains.

The structure of the three pigments were elucidated by 1H-NMR and mass spectrometry. The 1H-NMR spectra of the pigments from the two strains were identical in all details.

The 1H-NMR spectrum of pigment 3 immediately reflected the C3-symmetry of its structure. The spectrum consisted of a carotenoid part which was practically identical with that of zeaxanthin (Schwieter et al., 1969; Englert 1982). Only minor shift changes were found, as expected, at some signals of the aliphatic protons of the 3-substituted cyclohexene end groups. Thus, small upfield shifts were seen for H-4,4'eq (from 2.388 ppm in zeaxanthin to 2.332), for H-4,4'ax (2.045 to ca 2.032); small downfield shifts occurred at H-2,2'eq (1.771-1.865) and H-2,2'ax (1.478-1.553). The axial protons H-3,3' absorbed similarly as in zeaxanthin at ca 3.98 ppm. The shape of all these multiplets and hence the position of the spectrum of zeaxanthin 3-p-glucosides of E. herbicola and from E. coli carrying the carotenoid gene cluster of the former are the first examples of ring glucosylation in nature. Furthermore, we have demonstrated the glycosidic-linkage by analogy for the other pigments.

The 1H-NMR of pigment 1 indicated the presence of only one glucose moiety with the same chemical shifts as in pigment 3. The carotenoid part of the spectrum can be additively composed from a zeaxanthin half (as in pigment 3) with identical shifts and further signals, typical for β-carotene (Vecchi et al., 1981). These signals were at 1.029 (6H, s, H-16', H-17'), ca 1.47 (2H, m, H-2'), ca 1.62 (2H, m, H-3'), 1.719 (3H, s, H-18'), 1.974 (9H, s, H-19' and H-20' together with H-20), 6.137 and 6.177 (ca 2H, AB-type, J=15.8 Hz, H-8' and H-7'), 6.154 (ca 2H, d, J=11 Hz, H-10' and H-10), 6.357 (1H, d, J=14.7 Hz, H-12'), 6.658 (ca 1H, dd, J=14.7, 11 Hz, H-11'). The remaining signals of H-14'=(H14) and H-15'=(H15) were as in pigment 3 at 6.255 and 6.636.

The mass spectrum of pigment 1 had relevant peaks at m/z 882 (M, 40), 790 (25), 724 (3), 331 (10), 209 (25), 169 (100), 127 (33), 109 (60), 91 (30), 81 (16) and 43 (50).

The 1H-NMR of pigment 2 consisted of a superposition of the spectrum of zeaxanthin 3-β-glucoside, with all shifts and coupling constants preserved as in pigment 1, and further signals which prove the presence of a 3-acetylated zeaxanthin half of the molecule (Englert, 1982). The signals belonging to this part were assigned at 1.076 and 1.107 (3H each, s, H-16' and H-17'), 1.584 (1H, t, J=12.3 Hz, H-2'ax), 1.725 (6H, s, H-18' together with H-18), 1.781 (1H, dd, J=12, 3.5, 1.5 Hz, H-2'eq), 1.969 (ca 3H, s, H-19'), 1.974 (ca 6H, s, H-20' together with H-20), 2.053 (3H, s, 3′-OAc), ca 2.112 (1H, m, H-4'ax), 2.450 (1H, dd, J 17.5 Hz H-4'eq), ca 5.06 (1H, m, H-3'ax), 6.092 and 6.126 (2H, AB-type, J=16 Hz, H-7' and H-8'), 6.165 (1H, d, J=11, H-10'), 6.372 (1H, d, J=14.8 Hz, H-12'), 6.644 (ca 1H, dd, J ca 15 and 11 Hz, H-11'). The remaining protons H-14'=(H-14) and H-15'=(H-15) were identified as in all other cases at 6.261 and ca 6.64 ppm.

The mass spectrum of pigment 2 showed prominent peaks at 940 (M, 20), 880 (8), 848 (7), 788 (7), 722 (3), 533 (3), 331 (13), 175 (25), 169 (100), 145 (30), 119 (45), 109 (55), and 43 (50).

**DISCUSSION**

In carotenoids, glycosylation is seldom found at ring hydroxyls. To our knowledge the only examples for such a substitution are the zeaxanthin rhamnosides of Corynebacteria (Schneider et al., 1973; Nybraaten and Liaaen-Jensen, 1974; Hertzberg et al., 1976), whereas glycosylated in-chain hydroxyls are more common in nature (for a review see Straub, 1987). The β-glucosides of zeaxanthin and β-cryptoxanthin described here from the parent strain of E. herbicola and from E. coli carrying the carotenoid gene cluster of the former are the first examples of ring glucosylation in nature. Furthermore, we have demonstrated the glycosidic-linkage
and absolute configuration of the newly identified zeaxanthin diglucoside. A chemical partial synthesis of zeaxanthin mono- and diglucosides have been reported (Pfander and Hodler, 1974) from zeaxanthin and α-acetobromogluco-ses. A carotenoid biosynthesis pathway for E. herbicola is outlined in Fig. 3 based on our analysis of the intermediates accumulated. We suggest that the same pathway also functions in E. coli carrying the E. herbicola carotenoid biosynthesis genes. As E. coli is capable of synthesizing GGPP but not carotenoids (Sherman et al., 1989), the E. herbicola carotenoid biosynthesis genes presumably encode all of the enzymes necessary to convert GGPP to the zeaxanthin glucosides.

The qualitative carotenoid analysis presented here also reveals that the expression of the carotenoid genes in the E. coli HB101(pPL376) cells results in similar carotenoid biosynthesis products as in the parent E. herbicola strain, thereby maintaining features of cis/trans isomerism. The differences in the relative amounts of the individual carotenoid species may be due to growth conditions, but this was not further investigated. The amount of carotenoid, relative to protein, was surprisingly found always to be about 2-fold higher in E. coli HB101(pPL376) than in Erwinia, cf. Tables 1 and 2.

Since the carotenoid pattern is identical in both organisms, the genes for all enzymes required for carotenoid biosynthesis in E. herbicola must be localized on the 12.4 kb DNA fragment from this organism carried by E. coli HB101(pPL376). This construct provides an ideal model system for the study of carotenoid biosynthesis due to the ease of manipulation of genes in E. coli.

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