Functional Complementation in *Escherichia coli* of Different Phytoene Desaturase Genes and Analysis of Accumulated Carotenes

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Three different phytoene desaturase genes, from *Rhodobacter capsulatus, Erwinia uredovora,* and *Synechococcus* PCC 7942, have been functionally complemented with a gene construct from *E. uredovora* which encodes all enzymes responsible for formation of 15-*cis* phytoene in *Escherichia coli.* As indicated by the contrasting reaction products detected in the pigmented *E. coli* cells after co-transformation, a wide functional diversity of these three different types of phytoene desaturases can be concluded. The carotenes formed by the phytoene desaturase from *R. capsulatus* were *trans*-neurosporene with three additional double bonds and two *cis* isomers. Furthermore, small amounts of three ζ-carotene isomers (2 double bonds more than phytoene) and phytofluene (15-*cis* and all-*trans* with + 1 double bond) were detected as intermediates. When the subsequent genes from *E. uredovora* which encode for lycopene cyclase and β-carotene hydroxylase were present, neurosporene, the phytoene desaturase product of *R. capsulatus,* was subsequently converted to the monocyclic β-zeacarotene and its monohydroxylation product. The most abundant carotene resulting from phytoene desaturation by the *E. uredovora* enzyme was *trans*-lycopene together with a *cis* isomer. In addition, bisdehydrolycopene was also formed. The reaction products of *Synechococcus* phytoene desaturase were two *cis* isomers of ζ-carotene and only small amounts of *trans*-ζ-carotene including 15-*cis*. The I_{50} values for flurtamone and diphenylamine to inhibit phytoene desaturation were determined and differential inhibition was observed for diphenylamine.

Introduction

Phytoene is the first carotene in carotenoid biosynthesis. Depending on the diversity of the end products of the carotenogenic pathways a variable number of double bonds is introduced into this hydrocarbon. Desaturation of phytoene proceeds by a dehydrogenase-electron transferase mechanism [1]. The enzyme involved is feed-back regulated in various organisms [1, 2]. Furthermore, it has been shown with a range of *Synechococcus* mutants that phytoene desaturase is the rate-limiting step in the pathway [3].

Genes of phytoene desaturase have been sequenced from *Rhodobacter capsulatus* [4, 5], *Neurospora crassa* [6], and *Erwinia uredovora* [7]. They all share conserved amino acids sequences. Recently, a phytoene desaturase has been cloned from the cyanobacterium *Synechococcus* which lacks homology with the other phytoene desaturase genes mentioned above [8]. Molecular genetic studies with *Erwinia* species showed that the product of this phytoene desaturase is lycopene [7, 9]. In case of *Rhodobacter,* lycopene is not an intermediate in this organism. Furthermore, it has been concluded from biochemical studies of organisms with oxygenic photosynthesis like cyanobacteria that phytoene desaturase catalyzes the conversion of phytoene to ζ-carotene only [10]. In addition, the sensitivity of phytoene desaturase from the three prokaryotic organisms to various inhibitors is different [9, 11]. Therefore, the properties of the different phytoene desaturase gene products should be compared. In this study we analyzed the carotenes formed in *E. coli* transformed with a plasmid containing a phytoene-synthesizing gene construct together with a plasmid carrying the phytoene desaturase genes of either *Erwinia, Rhodobacter,* or *Synechococcus,* and the effects of inhibitors upon the desaturation catalyzed by these different desaturases.

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Materials and Methods

Bacterial strains, plasmids and growth conditions

E. coli strain JM101 was used as a host for the plasmids pACCRT-EB, pACCAR25delD, pCAR-D, pGABX2, and pPDSdel35. The plasmid pACCRT-EB is derived from the pCAR16 plasmid [7]. For its construction a *Bst*EII (1235)-*Eco*52I (4926) fragment was removed from pCAR16, and a 2.3 kb *Asp*718 (*Kpn*I)-*Eco*RI fragment carrying *crtE* and *crtB* of *E. uredovora* was isolated and inserted into the *Eco*RV site of pACYC184 [12, 13], as shown in Fig. 1. A 6.5 kb *Asp*718 (*Kpn*I)-*Eco*RI fragment of pCAR25delD [7], in which only the *crtI* gene was removed, was inserted into the *Eco*RV site of pACYC184 to yield pACCAR25delD. The pCAR-D plasmid carrying only the *E. uredovora crtI* gene which was cloned as the *Sna*BI (3497)-*Mlu*I (5379) fragment of plasmid pCAR16 [7] in the pUC18 vector. The pGABX2 plasmid resulted from the insertion of the *Bgl*I-*Xho*I restriction fragment from the plasmid pRPS404 [14] into pBR322, as described in ref. [4]. The pPDSdel35 contains the complete phytoene desaturase encoding region of the *pds* gene from the cyanobacterium *Synechococcus* PCC7942 [8] plus additional 27 nucleotides upstream from the translation start site cloned in *Kpn*I-*Bam*HI sites of pBLUESCRIPT KS. This creates an in-frame fusion of *pds* with *lacZ* so that the first 19 amino acids are of *lacZ*. The *E. coli* strain JM101 and the corresponding transformants were grown in L broth in the dark as described by Maniatis *et al.* [15], ampicillin (100 μg/ml), chloramphenicol (30 μg/ml), isopropyl-β-D-thio-galactopyranosid (0.5 mM) were added as required. The inhibitors used were the herbicide flurtamone [5-methylamino-2-phenyl-4-(3-trifluromethylphenyl)-3(2*H*)furanone] from Chevron Chemical Company [16], the pyrimidine compound KM 143-958 from Sandoz AG, Basle, Switzerland and diphenylamine from Merck, Darmstadt, Germany.

Enzymes and DNA techniques

All restriction enzymes, Klenow enzyme and DNA ligase were purchased from Boehringer, Mannheim. The reactions were performed according to Maniatis *et al.* [15].

Carotenoid extraction and HPLC analysis

Carotenoids (carotenes and hydroxylated products) from the different *E. coli* transformants were isolated from the pelleted freeze-dried cells by extraction with methanol containing 6% KOH for 20 min at 60 °C, with centrifugation and re-extraction of the pellet with acetone. The combined extracts were partitioned into diethylether/petrol (b.p. 35–80 °C) (1:9, v/v). The diethylether/petrol phase was either used to determine the optical density at the corresponding wavelength or evaporated to dryness. After resuspension in methanol, the carotenoids were separated by HPLC. A Spherisorb ODS-1 5 μ column was used with acetonitrile/methanol/2-propanol (85:10:5, v/v/v) as eluent [16]. The carotenoids were separated and detected with a Waters 994 diode array detector and spectra were directly recorded from the elution peaks. For comparison, the following standards were isolated as previously described [17]: *trans*-phytoene, *trans*-ζ-carotene, *trans*-neurosporene, and *trans*-lycopene. In addition, β-zeacarotene was obtained from *Scenedesmus* cells treated with a ζ-carotene desaturase inhibitor [18] and 15-*cis* as well as *trans*-phytofluene after norflurazon treatment [19].

Results

For the complementation studies with phytoene desaturases from different organisms, two different plasmids in pACYC184 have been constructed (Fig. 1). The resulting pACCRT-EB carries the *crtE* and *crtB* genes from *E. uredovora* which mediate the formation of 15-*cis* phytoene in *E. coli* [7]. Plasmid pACCAR25delD contains the complete carotenogenic gene cluster from *E. uredovora* for the synthesis of glucosylated zeaxanthin [7], except for the phytoene desaturase gene which was deleted.

The complementations in Fig. 2 were carried out by co-transformation of *E. coli* with the phytoene producing plasmid pACCRT-BE and a plasmid with the gene of phytoene desaturase. HPLC analysis of the resulting carotenes is shown in Fig. 2 and the absorbance spectra of the separated compounds are presented in Fig. 3. The phytoene desaturase from *Synechococcus* formed three isomers with a ζ-carotene spectra (nos. 7, 8, 9 in Fig. 2A). No. 7 was present only in trace amounts

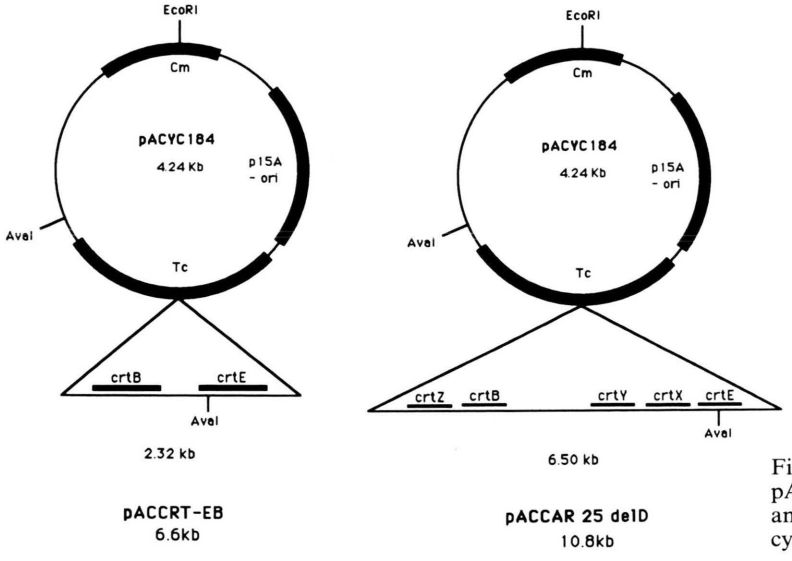

Fig. 1. Construction of plasmids pACCRT-EB and pACCR25delD. Cm and Tc show chloramphenicol and tetracyclin resistance genes.

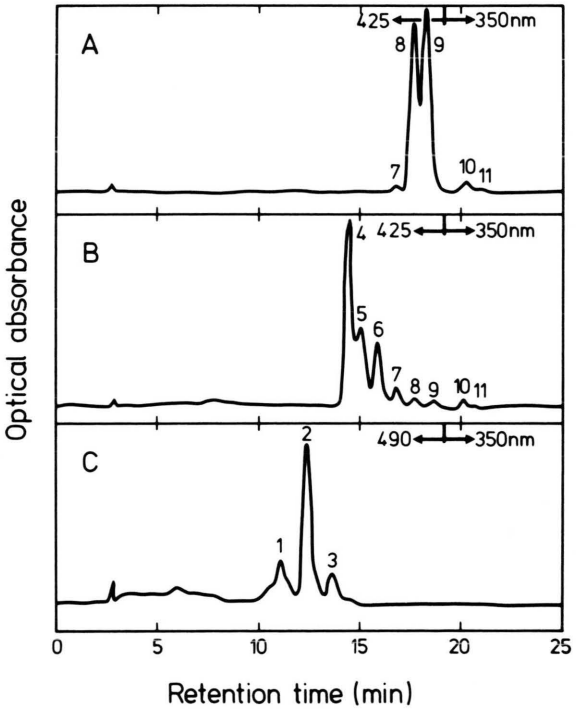

Fig. 2. HPLC separation of the reaction products obtained after complementation of phytoene desaturases from *Synechococcus* PCC7942 (A), *Rhodobacter capsulatus* (B), and *Erwinia uredovora* (C) in *E. coli*. The absorbance was first recorded at 425 and then at 350 nm. The following carotenes were identified: 1, bisdehydrolycopene; 2, all-*trans* lycopene; 3, *cis* lycopene; 4, all-*trans* neurosporene; 5 and 6, *cis* neurosporene; 7, all-*trans* ζ-carotene; 8 and 9, *cis* ζ-carotene; 10, all-*trans* phytofluene; 11, 15-*cis* phytofluene.

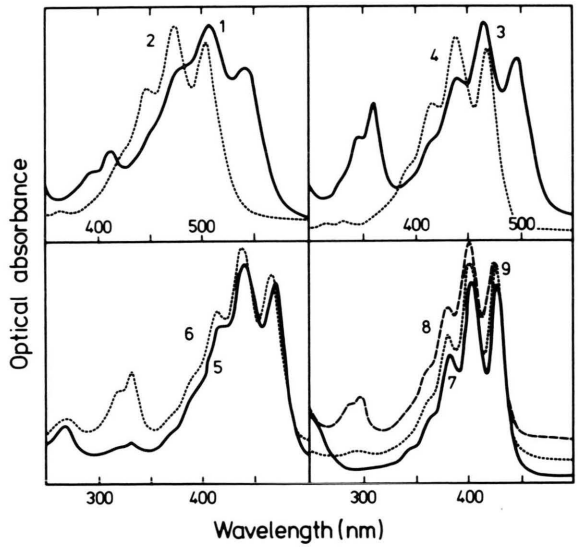

Fig. 3. Optical absorbance spectra of carotenes from the elution peak of HPLC separation (see Fig. 2 for peak numbering).

(less than 5% of all ζ-carotenes). This was identified as all-*trans* ζ-carotene from its spectrum as well as by co-chromatography with an authentic standard. The main absorption maxima of the three isomers were at 403, 400, and 402 nm. The ζ-carotene isomers nos. 8 and 9 exhibit more or less pronounced *cis* peaks at 298 nm. Their relative heights to the main absorbance maximum are 0.23 and 0.05, respectively. In addition to ζ-carotene, two colorless carotenes were formed (nos. 10 and 11) which co-chromatograph with all-*trans* (retention time of 20 min) and 15-*cis* phytofluene (retention time of 21 min) and also show the spectra of both phytofluene isomers [19].

E. coli co-transformed with the *R. capsulatus* phytoene desaturase synthesized ζ-carotene and phytofluene isomers (nos. 7 to 11) formed by the *Synechococcus* phytoene desaturase, but only in small amounts (Fig. 2B). Instead, the major carotenes formed were 3 isomers of neurosporene (nos. 4, 5, and 6) with absorbance maxima at 440, 439, or 436 nm. No. 4 co-chromatographs with all-*trans* neurosporene. A pronounced *cis* peak at 330 nm was observed for neurosporene no. 6 with a relative height to the main absorbance band of 0.38.

The reaction products of the phytoene desaturase from *E. uredovora* in *E. coli* were two compounds with a lycopene spectrum (nos. 2 and 3) exhibiting main absorbance maxima at 473 and 468 nm, respectively. No. 2 co-chromatographs with all-*trans* lycopene. Compound no. 3 shows a strong *cis* peak at 364 nm with a relative height of 0.59. Another carotene (no. 1) with maxima at 483, 509, and 543 nm was present. By comparison of its absorbance maxima [20] and by its higher polarity than lycopene as indicated by its lower retention time, this carotene was identified as bisdehydrolycopene.

When complementation of the *R. capsulatus* phytoene desaturase gene was carried out not only with those genes responsible for phytoene synthesis but also with genes encoding for lycopene cyclase and β-carotene hydroxylase the same carotenes accumulated as were found with the transformed *E. coli* in which the latter two genes were missing. However, 5 new carotenoids were evident (nos. 12, 13, 14, 15, and 16 of Fig. 4A) with similar spectra. They exhibit maxima of about 409, 430, and 455 nm. Nos. 14 to 16 were identified as β-zeacarotene isomers by their spectra [20] and co-chromatography with extracts of *Scenedesmus* treated with the ζ-carotene desaturase inhibitor KM 143-958 [18]. These cells accumulated β-zeacarotene. As indicated by similar spectra and by their lower retention times of 11.8 (no. 12) and 12.3 min (no. 13) the two carotenoids should be polar β-zeacarotene derivatives. For no. 12 mass spectroscopy gave prominent ions at *m/e* 554 and 536.

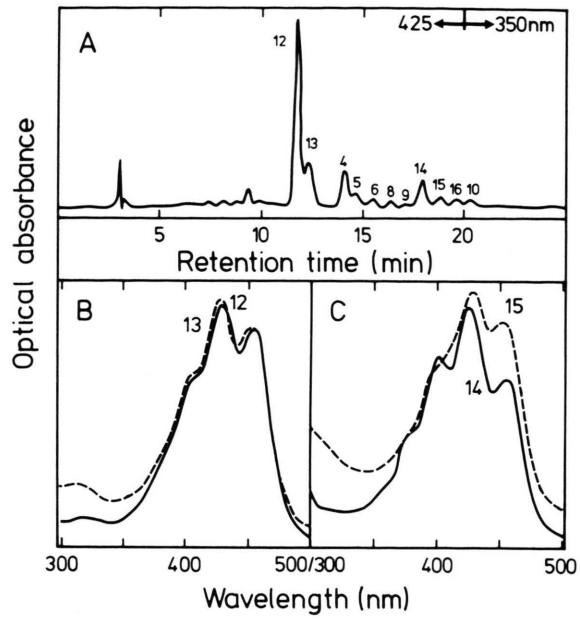

Fig. 4. HPLC separation (A) and optical absorbance spectra (B, C) of carotenoids formed in complemented *E. coli* with a complete carotenogenic gene cluster from *Erwinia uredovora* in which the phytoene desaturase was deleted and functionally replaced by the *Rhodobacter capsulatus* phytoene desaturase gene. The following carotenoids in addition to those in Fig. 2 were identified: 12 and 13, hydroxy-β-zeacarotene; 14, 15, 16, β-zeacarotene isomers.

The sensitivity of the three phytoene desaturases against the two inhibitors flurtamone and diphenylamine (DPA) has been determined (Fig. 5). Dixon plots of inhibitor concentration *versus* reciprocal product concentration (as % of untreated control) were used to determine I_{50} values. Inhibition of phytoene desaturase from all three species was observed in the presence of flurtamone (Fig. 5A) with an average I_{50} value of about 4 μM.

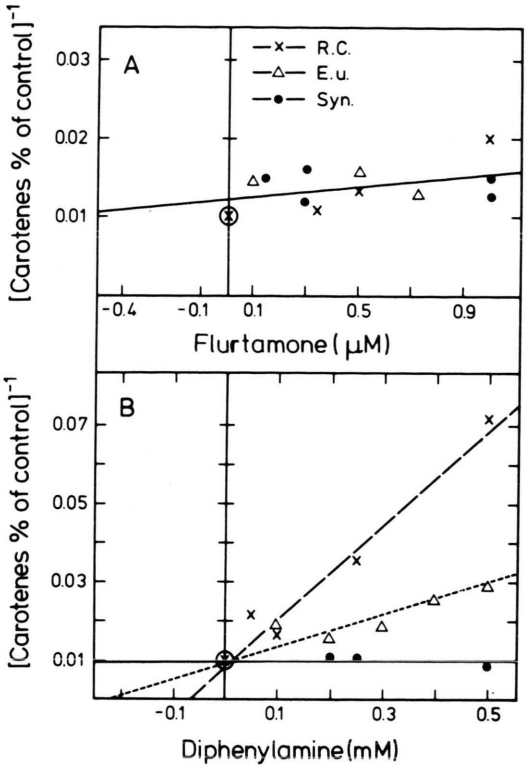

Fig. 5. Dixon plots for determination of I_{50} values for inhibition of the formation of phytoene desaturase products in *E. coli* transformed with phytoene desaturase genes from different organisms (−x− *R. capsulatus;* −△− *E. uredovora;* −●− *Synechococcus*). The inhibitors used were flurtamone and diphenylamine.

In case of DPA, inhibition of phytoene desaturation was negligible for the *Synechococcus* enzyme. For phytoene desaturation in *E. coli* cells carrying either the *R. capsulatus* or *E. uredovora* phytoene desaturase gene, I_{50} values of 65 and 215 μM were determined, respectively.

Discussion

Functional complementation of sequences of a biosynthetic pathway by co-transformation of *E. coli* with plasmids carrying different genes is a convenient technique either for establishing the function of a gene or for the study of reaction products of enzymes which have not been isolated as yet. This type of experiments was carried out for phytoene desaturases from three different organisms. Complementation experiments with the *pds* gene from *Synechococcus* [8] gave definite evidence that this gene, which is structurally distinct from the *crtI* genes of *R. capsulatus* and *E. uredovora* [4, 5, 7] encodes for phytoene desaturase. A gene cloned by hybridization of *Synechocystis* DNA with the *R. capsulatus crt I* gene [21] did not result in formation of any colored carotene after complementation.

There is a dispute over the recent years on the number of desaturase enzymes involved in the formation of lycopene from phytoene [10]. Contrasting conclusions were drawn from several groups working with different organisms. In our investigation, it could be demonstrated that the enzymes from *Synechococcus, R. capsulatus,* and *E. uredovora* differ in the number of desaturation steps. The *Synechococcus* enzyme introduces two double bonds symmetrically at C-11 and C-11′ into phytoene yielding ζ-carotene (Fig. 2). Trace amounts of the intermediate phytofluene with only one additional double bond could also be detected. The phytoene desaturase from *R. capsulatus* is a three-step enzyme introducing a further double bond in ζ-carotene at C-7 to give neurosporene. Additionally, the one- and two-step precursors phytofluene and ζ-carotene are also present in low concentrations. The phytoene desaturase from *E. uredovora* very efficiently carries out four desaturation steps (at positions C-7, C-7′, C-11, C-11′) forming lycopene as the major product. Furthermore, bisdehydrolycopene (= 3,4,3′,4′-tetradehydrolycopene) with 6 double bonds more than phytoene is also synthesized. This indicates a potential of this phytoene desaturase to dehydrogenate phytoene to its maximal conjugated system. However, this carotene and corresponding reaction products are absent both from *Erwinia* [22] and *E. coli* carrying the complete gene cluster including lycopene cyclase [7]. In a complementation experiment involving the phytoene desaturase gene from *R. capsulatus* and from *N. crassa,* the enzyme from the latter fungus converted phytoene or neurosporene into 3,4-dehydrolycopene which indicates that the *N. crassa* phytoene desaturase can maximally carry out 5 desaturation steps [23].

With respect to their end products, all three phytoene desaturases are functionally different enzymes. One consequence of ζ-carotene being formed as the reaction product of the *Synechococcus* phytoene desaturase is the need for this organ-

ism to contain an additional ζ-carotene desaturase instrumental to form lycopene. This has been concluded previously from biochemical work with mutants and from inhibitor studies [10] and is confirmed by the results presented. The amino acid sequence deduced from the gene sequence is quite similar for the enzyme from *R. capsulatus* and *E. uredovora* [4, 7] whereas the composition of the *Synechococcus* enzyme is completely different [8]. The diversity of the two types of phytoene desaturase is also reflected by their differential inhibitor sensitivity. The enzymes from *R. capsulatus* and *E. uredovora* are inhibited by diphenylamine whereas the enzyme from *Synechococcus* is insensitive (Fig. 4).The I_{50} values (around 4 μM) for inhibition of carotenoid formation by the inhibitor flurtamone are about the same for all three phytoene desaturases. However, in *Synechococcus* cells a value of 3×10^{-8} M was obtained [16]. This discrepancy may be due to restricted uptake of this herbicide by *E. coli* cells. Other herbicidal phytoene desaturase inhibitors, like fluridone, showed also very low inhibition of ζ-carotene formation in *E. coli* containing the *Synechococcus* phytoene desaturase gene.

Another topic of disagreement in the literature are the isomerization steps in the carotenoid pathway from 15-*cis* phytoene to *e.g.* all-*trans* β-carotene as the major compound [10]. In complemented *E. coli*, carotene intermediates accumulate up to 3 different isomers (Fig. 3A). In case of phytofluene there is more of the all-*trans* than the 15-*cis* form. Two *cis* isomers of ζ-carotene which are spectroscopically different from the 15-*cis* isomer [24] dominate. When neurosporene is accumulated, it is mainly the all-*trans* isomer (Fig. 2B, no. 4). From the spectral reference data available [25] no. 5 may be a 5 or 9′ neurosporene. No. 6 seems to be different from all other neurosporene isomers generated by photoisomerization. The lycopene is about 85% all-*trans*. The one additional *cis* isomer which was also found in a lycopene cyclase deletion mutant [9] shows a *cis* peak which in size was close to the one of 15-*cis* lycopene (relative height of 0.7; H. J. Maier and K. Bernhard, Basle, personal communication). According to the very high *cis* peak, compound no. 3 might be a 13-*cis* lycopene isomer [26].

The relative amounts of *cis* isomers of carotenes are more or less the same when they are accumulated even from phytoene desaturases with different end products. Obviously, there is a gradual quantitative shift from formation of *cis* carotene isomers to *trans* forms with increase of their conjugated system. An extension of π electron delocalization in the carotene molecules may facilitate the detachment of a reaction product with a *trans* central double bond from the desaturases.

The complementation study with the gene of *R. capsulatus* phytoene desaturase which forms the unsymmetrical neurosporene molecule and the genes of lycopene cyclase and β-carotene hydroxylase introduced simultaneously, illustrates the substrate requirements of the latter enzymes. In addition to neurosporene and precursors, high and low polarity compounds with β-zeacarotene-like spectra are formed as major products. Compounds nos. 14 to 16 are β-zeacarotene isomers. This direct cyclization product of neurosporene can be accumulated in algae in which neurosporene desaturation is blocked by inhibitors [18]. Compounds nos. 12 and 13 were identified by their chromatographic polarity and mass spectroscopy as hydroxy-β-zeacarotene. This carotenoid has once before been identified in *Flavobacterium* [27]. The occurrence of the two carotenoids in the complemented *E. coli* demonstrates that either lycopene cyclase or β-carotene hydroxylase recognize half of the carotene molecule as substrate when it carries a conjugated system including a C-7 double bond or a β-ionone ring, respectively.

Acknowledgements

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