ELSEVIER

Journal of Biotechnology 97 (2002) 51–58

JOURNAL OF Biotechnology

www.elsevier.com/locate/jbiotec

Heterologous production of two unusual acyclic carotenoids, 1,1′-dihydroxy-3,4-didehydrolycopene and 1-hydroxy-3,4,3′,4′-tetradehydrolycopene by combination of the *crtC* and *crtD* genes from *Rhodobacter* and *Rubrivivax*

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Received 27 September 2001; received in revised form 28 February 2002; accepted 15 March 2002

Abstract

Acyclic hydroxy carotenoids were produced from lycopene and 3,4-didehydrolycopene in *Escherichia coli* by combining different carotenogenic genes including the carotene hydratase gene *crtC* and the carotene 3,4-desaturase gene *crtD*. The genes originated either from *Rhodobacter* species or *Rubrivivax gelatinosus*. It was shown that the product of *crtD* from *Rubrivivax* unlike the one from *Rhodobacter* is able to convert 1-HO-3,4-didehydrolycopene to 1-HO-3,4,3′,4′-tetradehydrolycopene (= 3,4,3′,4′-tetradehydro-1,2-dihydro-ψ,ψ-caroten-1-ol). Thus, only when the desaturase from *Rubrivivax* is expressed can this novel carotenoid be obtained. In the presence of *crtC* from *Rubrivivax*, another carotenoid 1,1′-$(HO)_2$-3,4-didehydrolycopene (= 3,4-didehydrolycopene-1,2,1′,2′-tetrahydro-ψ,ψ-caroten-1,1′-diol) not found in a non-transgenic organism before is formed in *E. coli*. Its accumulation under these conditions and its absence when *crtC* from *Rubrivivax* is replaced by the corresponding gene from *Rhodobacter* is discussed. The function of the different *crtC* and *crtD* genes in the pathway leading to the individual carotenoids is outlined. Since 1,1′-$(HO)_2$-3,4-didehydrolycopene could not be produced in substantial amounts and 1-HO-3,4,3′,4′-tetradehydrolycopene has not been described before, their structural characteristics were determined for the definite assignment of their identity. This included spectral properties, determination of relative molecular mass as well as the number of hydroxy groups by mass spectroscopy and NMR spectroscopy for 1,1′-$(HO)_2$-3,4-didehydrolycopene.

Keywords: Carotenoid production; Combinatorial biosynthesis; *Escherichia coli* transformants; 1,1′-$(HO)_2$-3,4-didehydrolycopene (= 3,4-didehydrolycopene-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol); 1-HO-3,4,3′,4′-tetradehydrolycopene (= 3,4,3′,4′-tetradehydro-1,2-dihydro-ψ,ψ-caroten-1-ol)

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0168-1656/02/$ - see front matter
PII: S0168-1656(02)00055-X

1. Introduction

Carotenoids are effective antioxidants protecting membranes and lipophilic cell constituents (Krinsky, 1994). Evidence is accumulating that certain carotenoids play an important role in the prevention of cancer and cardiovascular diseases (Gerster, 1993; Mayne, 1996). For example, evidence was obtained that lycopene supplementation reduced the growth of prostate cancer (Kucuk et al., 2001) and that a low lycopene serum level increased the risk of coronary events or stroke (Rissanen et al., 2001). The main commercial importance of carotenoids is as food and feed additives (Nelis and de Leenheer, 1991). Only a few carotenoids like ß-carotene and astaxanthin are produced in a well-established chemical process (Bernhard, 1989), others are available from natural sources (Nonomura, 1989). However, the supply of different carotenoid derivatives is limited. Biosynthesis in transgenic microorganisms is an alternative to obtain carotenoids of various chemical structures (Sandmann et al., 1999). Fungi (Yamano et al., 1994; Shimada et al., 1998) and bacteria (Misawa et al., 1991; Ruther et al., 1997) have been used for this purpose. Among them, *Escherichia coli* is the most versatile host. By its transformation with a combination of carotenogenic genes from prokaryotes and eukaryotes, it was possible to produce rare and novel carotenoids (Albrecht et al., 1997a). The *crtC* and *crtD* genes from *Rhodobacter* species are involved in the biosynthesis of acyclic hydroxy carotenoids with an extended conjugated double bond system which exhibited superior antioxidative properties (Albrecht et al., 2000). Functional screening of the products of homologous genes from other bacteria may reveal similar enzymes with extended catalytic properties.

In the present publication we demonstrate that highly homologous genes from closely related photosynthetic bacteria encode enzymes which differ in their product specificities. We used the *crtC* and *crtD* genes from *Rubrivivax gelatinosus* for the biosynthesis of rare 1,1′-$(HO)_2$-3,4-didehydrolycopene (Albrecht et al., 2000) and novel 1-HO-3,4,3′,4′-tetradehydrolycopene.

2. Material and methods

2.1. Organism, cultivation and plasmids

E. coli strain JM101 was the host for various combinations of plasmids. The transformants were cultivated in LB medium supplied with appropriate antibiotics in concentrations of 100 μg ml^{-1} ampicillin, 34 μg ml^{-1} chloramphenicol, 25 μg ml^{-1} tetracycline and/or 25 μg ml^{-1} kanamycin (Sambrook et al., 1989). Isopropylthiogalactoside (0.5 mM) was added to the cultures 18 h after inocculation. Growth was for 48 h at a temperature of 28 °C. Plasmids pACCRT-EBI_{Eu} and pACCRT-EBal1 mediate the formation of lycopene and 3,4-didehydrolycopene, respectively in non-carotenogenic *E. coli* (Albrecht et al., 2000). In each case, the *crtE* gene encoding a geranylgeranyl pyrophosphate synthase and the *crtB* gene both from *Erwinia uredovora* encoding phytoene synthase enable the formation of phytoene (Sandmann and Misawa, 1992). The products of genes $crtI_{Eu}$ from *E. uredovora* and *al-1* from *Neurospora crassa* are desaturases which convert phytoene to lycopene or 3,4-didehydrolycopene, respectively. Plasmid pUCCRT-C_{Rc} carrying the hydratase gene *crtC* from *Rba. capsulatus* (Albrecht et al., 1997a), and pQECRT-D_{Rs} with the carotene 3,4-desaturase gene *crtD* from *Rba. sphaeroides* have been previously described (Albrecht et al., 1997b). The gene *crtD* from *Rvi. gelatinosus* alone is part of plasmid pSO53 and in combination with *crtC* of pSO50 (Ouchane et al., 1997).

2.2. Extraction, separation and quantitation of carotenoids

Pigments were extracted from freeze-dried cells with acetone at a temperature of 50 °C for 20 min. The extract was partitioned against 10% ether in petrol. The upper phase was collected, the solvent evaporated and the residue dissolved in acetone. Carotenoids were routinely separated by HPLC on a 25 cm Nucleosil C18, 3 μm column (Macherey-Nagel, Düren, Germany) with acetonitrile/methanol/2-propanol (85:10:5 by volume) at a flow of 1 ml min^{-1} (Ruther et al., 1997). For

better separation of 1,1′-$(HO)_2$-3,4,3′,4′-tetradehydrolycopene from 1,1′-dihydroxy-3,4-didehydrolycopene a 25 cm Hypersil 5 μ HyPurity Elite C18 column (Knauer, Berlin, Germany) and the same solvent were used. Spectra were recorded on-line from the elution peaks with a Kontron 400 photodiode array detector. The amount of individual hydroxylated carotenoids was quantitated using non-hydroxylated carotenes with identical spectral characteristics together with spectral data compiled by Davis (1976). In the case of 1-HO-3,4,3′,4′-tetradehydrolycopene, an average extinction coefficient $A^{1\%}$ 1 cm of 2500 was used. Values are means of three determinations.

2.3. Identification of hydroxy carotenoids

All carotenoids were identified by comparison with authentic standards with the exception of 1-HO-3,4,3′,4′-tetradehydrolycopene and 1,1′-$(HO)_2$-3,4-didehydrolycopene. For their analysis, these two carotenoids were purified by column chromatography on silica 60 (Merck, Darmstadt, Germany) using hexane/chloroform mixtures of increasing polarity for elution. FD mass spectra were obtained using a Hitachi (Tokyo, Japan) Model M-2500 double-focusing gas chromatograph/mass spectrometer equipped with an FD apparatus as previously described (Takaichi, 1993). The number of hydroxy groups was determined by acetylation and trimethylsilylation (Takaichi, 1993), and the number of conjugated double bonds was deduced from the spectral properties (Takaichi and Shimada, 1992). ^{1}H-NMR spectra in $CDCl_3$ were obtained using a DRX400 spectrometer (Bruker, Germany).

3. Results and discussion

A biosynthetic pathway can be established in a heterologous host after all genes encoding the enzymes for the individual reactions have been introduced. When genes involved in a parallel pathway are combined, a chimeric pathway may lead to novel products depending on the enzyme recognition of only certain regions of the substrate molecule instead of the entire molecule. This strategy has been applied successfully for carotenoid biosynthesis (Sandmann et al., 1999). Among others, the *crtC* and *crtD* genes from *Rhodobacter* species encoding a hydratase adding water to C-1,2 of a ψ-carotene end group and a 3,4-desaturase dehydrating at C-3,4 of 1-hydroxy-1,2-dihydro-ψ end group, respectively, have been used before. In *Rhodobacter* cells, they participate in the biosynthesis of spheroidene (Takaichi, 1999). In *Rvi. gelatinosus* the products of *crtC* and *crtD* genes participate in the formation of hydroxyspheroidene and spirilloxanthin instead. For both genes, amino acid identities are between 40 and 50% and the similarities around 70%.

In Fig. 1 the carotenoids produced in *E. coli* carrying different combinations of *crtC* and *crtD* genes from *Rhodobacter* species and *Rubrivivax gelatinosus* were separated and identified. Identification was by their spectral characteristics and in the case of known carotenoids by co-chromatography with authentic standards. The structure of novel 1-HO-3,4,3′,4′-tetradehydrolycopene and also of 1,1′-$(HO)_2$-3,4-didehydrolycopene was further confirmed by mass spectroscopy (Table 1). Fig. 1A shows the HPLC separation of carotenoids from *E. coli* cells with a 3,4-didehydrolycopene background and transformed with *crtC* and *crtD* from *Rba. capsulatus* and *Rvi. gelatinosus*, respectively. Five major carotenoids accumulated and were identified as 1,1′-$(HO)_2$-3,4,3′,4′-tetradehydrolycopene (peak 1), 1-HO-3,4,3′,4′-tetradehydrolycopene (peak 3), 1-HO-3′,4′-didehydrolycopene (peak 4), demethylspheroidene (peak 6) and 3,4-didehydrolycopene (peak 7). In the *E. coli* transformant with the $crtD_{Rs}$ gene from *Rhodobacter* instead (Fig. 1B), carotenoids 1, 4, 6 and 7 but not carotenoid 3 were also present. In addition, 1,1′-$(HO)_2$-lycopene (peak 2) and 1-HO-lycopene peak (peak 5) were identified. In *E. coli* with a lycopene background carrying the $crtC_{Rg}$ and $crtD_{Rg}$ genes from *Rvi. gelatinosus*, 1,1′-$(HO)_2$-3,4-didehydrolycopene (peak 8) and 1-HO-3,4-didehydrolycopene (peak 9) were found in addition to carotenoids 1 and 7 (Fig. 1C). For their separation another column (Hypersil C18) was used in order to get a better resolution of dihydroxy compounds.

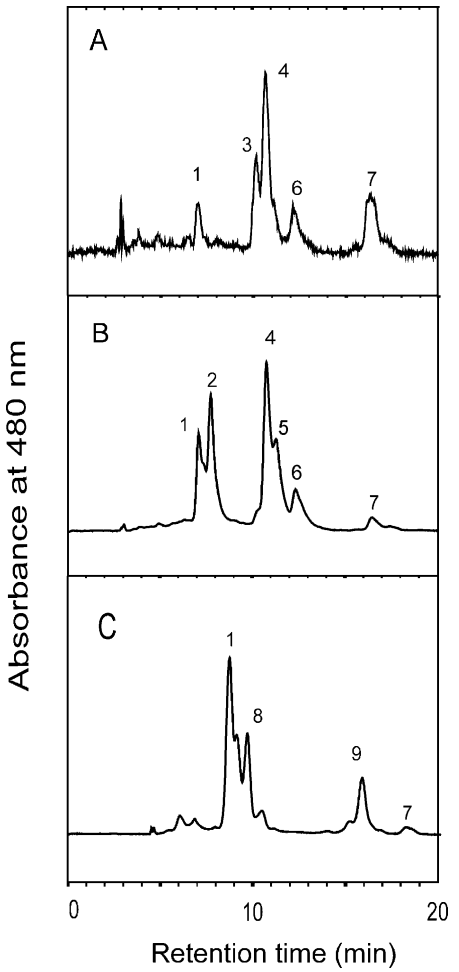

Fig. 1. HPLC separation of hydroxylated carotenoids. (A) *E. coli* with a background of 3,4-didehydrolycopene carrying plasmids with $crtC_{Rc}$ and $crtD_{Rg}$ from *Rubrivivax gelatinosus* and *Rhodobacter capsulatus*, respectively. (B) *E. coli* with a background of 3,4-didehydrolycopene carrying plasmids with $crtC_{Rc}$ and $crtD_{Rs}$ from *Rhodobacter* species. (C) *E. coli* with a background of lycopene carrying plasmids with $crtC_{Rg}$ and $crtD_{Rg}$ both from *Rubrivivax gelatinosus*. The numbers of the peaks correspond to 1,1′-$(HO)_2$-3,4,3′,4′-tetradehydrolycopene peak 1, 1,1′-$(HO)_2$-lycopene peak 2, 1-HO-3,4,3′,4′-tetradehydrolycopene peak 3, 1-HO-3′,4′-didehydrolycopene peak 4, 1-HO-lycopene peak 5, demethylspheroidene peak 6, 3,4-didehydrolycopene peak 7, 1,1′-$(HO)_2$-3,4-didehydrolycopene peak 8 and 1-HO-3,4-didehydrolycopene peak 9. Carotenoids are referred to in the text by the numbers indicated here. Separation in A. and B. was on a Nucleosil C18 and in C. on a Hypersil C18 column.

Table 1
Structural characteristics of the selected acyclic hydroxy carotenoids

	Carotenoids	
	1-HO-3,4,3′,4′-TDL	1,1′-$(HO)_2$-3,4-DDL[a]
Relative molecular mass	550	570
Number of hydroxy groups[b]	1	2
Absorption maxima (nm)[c]	466, 500, 535	305, 373, 456, 481, 513

1-HO-3,4,3′,4′-TDL, 1-hydroxy-3,4,3′,4′-tetradehydrolycopene (3), 1,1′-$(HO)_2$-3,4-DDL, 1,1′-dihydroxy-3,4-didehydrolyco-p-ene (8). Numbers refer to peak numbers of individual carotenoids in Fig. 1.

[a] This structure was confirmed by ^{1}H-NMR.

[b] Determined by the number of trimethylsilylation, acetylation was negative.

[c] Spectra were recorded in methanol.

Since 1,1′-$(HO)_2$-3,4-didehydrolycopene and 1-HO-3,4,3′,4′-tetradehydrolycopene are either rare or have not been isolated or synthesized before, their structural characteristics were determined for the definite assignment of their identity (Table 1). 1,1′-$(HO)_2$-3,4-didehydrolycopene (= 3,4-didehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1′-diol) has a relative molecular mass of 570 and gave two trimethylsilylation derivatives indicating the presence of two tertiary hydroxy groups. The absorbance maxima correspond to 12 conjugated double bonds (Takaichi and Shimada, 1992). The structure was further confirmed by ^{1}H-NMR showing the presence of a 1-hydroxy-1,2-dihydro-ψ and a 1-hydroxy-1,2-dihydro-3,4-didehydro-ψ end group. For 1-HO-3,4,3′,4′-tetradehydrolycopene (= 3,4,3′,4′-tetradehydro-1,2-dihydro-ψ,ψ-caroten-1-ol), the expected relative molecular weight of 550 and the presence of one tertiary hydroxy group was determined. The spectral properties were that of a carotenoid with 14 conjugated double bonds.

The carotenoid content of the *E. coli* transformants was quantitated in Table 2. In *E. coli* JM101/pACCRT-EBal1/pSO53/pUCCRT-C_{Rc} in which gene *al1* mediates carotene desaturation to 3,4-didehydrolycopene, most of the hydroxy

derivatives including 1-HO-3′,4′-didehydrolycopene (267 μg g^{-1} dw) and 1-HO-3,4,3′,4′-tetradehydrolycopene (101 μg g^{-1} dw) originate from this precursor. Smaller amounts of carotenoids are diverted from neurosporene to demethylspheroidene (85 μg g^{-1} dw) or from lycopene to 1,1′-(HO)$_2$-3,4,3′,4′-tetradehydrolycopene (99 μg g^{-1} dw) (Fig. 2). In *E. coli* JM-101 / pACCRT-EBal1 / pQECRT-D_{Rs} / pRKCRT-C_{Rc} where the *crtD* gene of *Rvi. gelatinosus* is replaced by the corresponding gene from *Rhodobacter*, the majority of carotenoids originate from hydroxylation of lycopene like 1,1′-(HO)$_2$-lycopene (121 μg g^{-1} dw), HO-lycopene (85 μg g^{-1} dw) and 1,1′-(HO)$_2$-3,4,3′,4′-tetradehydrolycopene (54 μg g^{-1} dw). The only hydroxylation product from 3,4-didehydrolycopene, 1-HO-3′,4′-didehydrolycopene, accumulated in amounts of only 92 μg g^{-1} dw. The other *E. coli* transformants JM101 / pACCRT-EBI_{Eu} / pSO53 / pUCCRT-C_{Rc} and JM101/pACCRT-EBI_{Eu}/pSO50 differ in the source of the *crtC* gene which is either from *Rba. capsulatus* or *Rvi. gelatinosus*. With the *Rhodobacter* gene carotenoid formation is limited at the conversion of 1-HO-3,4-didehydrolycopene. This carotenoid is highly accumulated (644 μg g^{-1} dw) and only small amounts (83 μg g^{-1} dw) are converted to 1,1′-(HO)$_2$-3,4,3′,4′-tetradehydrolycopene. With the *crtC* gene from *Rvi. gelatinosus* 1-HO-3,4-didehydrolycopene concentrations are comparably low (83 μg g^{-1} dw) and substantial amounts of the subsequent carotenoids in the pathway 1,1′-(HO)$_2$-3,4-didehydrolycopene (114 μg g^{-1} dw) and 1,1′-(HO)$_2$-3,4,3′,4′-tetradehydrolycopene (232 μg g^{-1} dw) are synthesized. Especially for 1,1′-(HO)$_2$-3,4-didehydrolycopene, formation of only trace amounts were reported when the *crtC* gene from *Rba. capsulatus* was used instead of *crtC* from *Rvi. gelatinosus* (Albrecht et al., 2000).

The biosynthetic pathway leading to the individual carotenoids in the *E. coli* transformants is outlined in Fig. 2. It is based on the carotenoids synthesized due to different gene combinations (Fig. 1), results from other complementation experiments (Albrecht et al., 1997a, 2000) and the in vitro substrate and product specificity of the enzyme encoded by *crtD* (Albrecht et al., 1997b; Steiger et al., 2000). The contribution of the individual gene products is indicated in Fig. 1. Although 3,4-didehydrolycopene is the desaturation end product of Al-1, CrtC can convert the intermediates neurosporene to demethylspheroidene and lycopene to hydroxy derivatives (Albrecht et al., 1997a). In the presence of the *crtD* gene from *Rvi. gelatinosus* a preference for the biosynthetic branch via 3,4-didehydrolycopene rather than via lycopene is evident (Table 2). The product of this gene has the potential to convert 1-HO-3′,4′-didehydrolycopene further on to 1-HO-3,4,3′,4′-tetradehydrolycopene whereas CrtD from *Rba. sphaeroides* is unable to catalyze this reaction. This corresponds to the negligible in vitro conversion of 1-HO-3′,4′-didehydrolycopene by the isolated enzyme from *Rba. sphaeroides*. The desaturation activity of CrtD from *Rba.*

Table 2
Plasmid combinations and carotenoid synthesis mediated by *crt* genes from *Rhodobacter* species and *Rubrivivax gelatinosus*

Background	Carotenoids (μg g^{-1} dry weight)						
(A) pACCRT-EBal1	(HO)$_2$-TDL	(HO)$_2$-L	HO-TDL	HO-DDL′	HO-L	DMS	DDL
+pSO53 (*crtD*$_{Rg}$)+pUCCRT-C_{Rc}	98.8	0	100.8	266.8	0	84.8	130.6
+pQECRT-D_{Rs}+pRKCRT-C_{Rc}	53.8	121.0	0	92.4	85.4	71.3	16.3
(B) pACCRT-EBI_{Eu}	(HO)$_2$-TDL	(HO)$_2$-DDL	HO-DDL	DMS	Lycopene		
+pSO53 (*crtD*$_{Rg}$)+pUCCRT-C_{Rc}	82.5	0	644.24	0	105.8		
+pSO50 (*crtC*$_{Rg}$, *crtD*$_{Rg}$)	232.1	113.9	83.2	13.1	0		

Abbreviations: (HO)$_2$-TDL, 1,1′-(HO)$_2$-3,4,3′,4′-tetradehydrolycopene (1); 1,1′-(HO)$_2$-L (2); HO-TDL, 1-HO-3,4,3′,4′-tetradehydrolycopene (3); HO-DDL′, 1-HO-3′,4′-didehydrolycopene (4); HO-L, 1-HO-lycopene (5); DMS, demethylspheroidene (6); DDL, 3,4-didehydrolycopene (7); (HO)$_2$-DDL 1,1′-(HO)$_2$-3,4-didehydrolycopene (8); HO-DDL, 1-HO-3,4-didehydrolycopene (9). Numbers refer to peak numbers of the carotenoids in Fig. 1. The function of the individual *crt* genes is specified in Section 2.

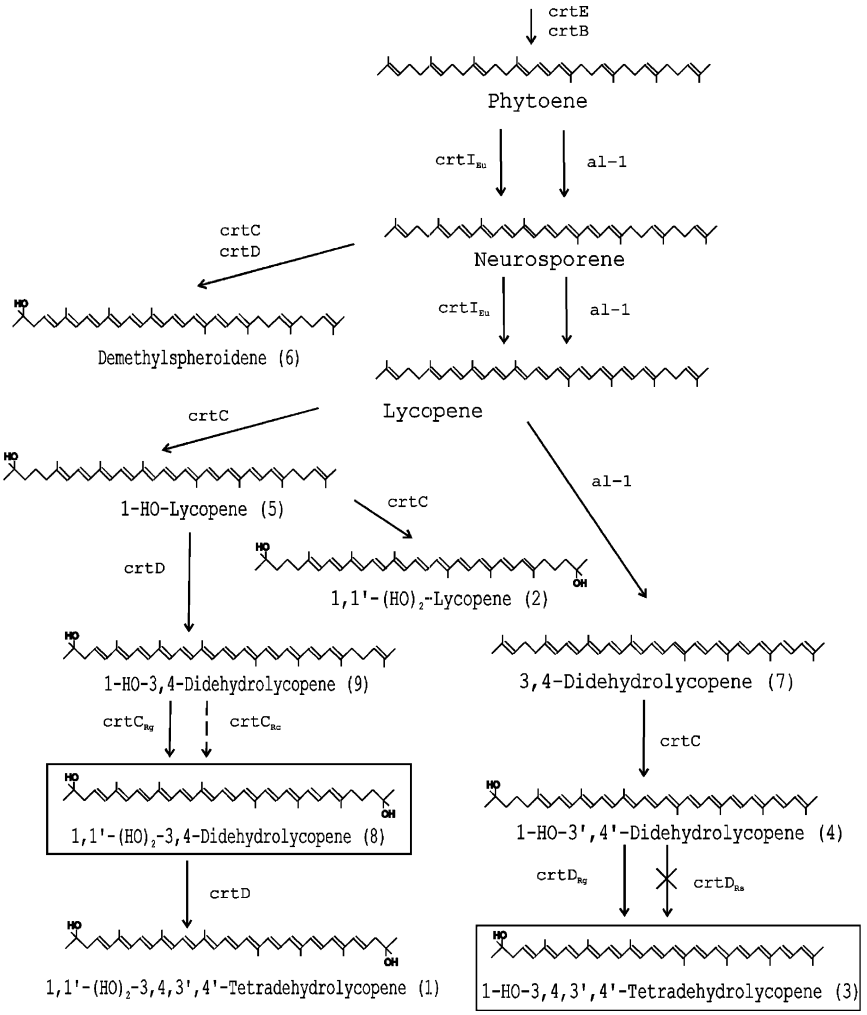

Fig. 2. Pathway of carotenoid formation in *E. coli* carrying the combination of indicated genes. Rare or novel carotenoids 1,1′-$(HO)_2$-3,4-didehydrolycopene (8) and 1-HO-3,4,3′,4′-tetradehydrolycopene (3) are boxed indicating that they are specifically synthesized in the presence of the *Rubrivivax gelatinosus* gene products of $crtD_{Rg}$ and $crtC_{Rg}$, respectively. Numbers refer to peak numbers of individual carotenoids in Fig. 1. Dashed arrow indicates conversion of only trace amounts.

sphaeroides which synthesizes 1-HO-3,4,3′,4′-tetradehydrolycopene may pull the metabolic flow into the 3,4-didehydrolycopene branch of the pathway. As indicated by the lack of both desaturases for substantial conversion of 1,1′-$(HO)_2$-lycopene (Albrecht et al., 1997b; Steiger et al., 2000), its formation should be a dead end in the combined pathway in *E. coli*.

When $crtD_{Rg}$ was combined with *crtC* either from *Rvi. gelatinosus* or *Rba. capsulatus* a difference in the accumulation of 1-HO-didehydrolycopene and its conversion to 1,1′-$(HO)_2$-didehydrolycopene and further on to 1,1′-$(HO)_2$-tetradehydrolycopene was found. Obviously, the $crtC_{Rc}$ gene product has only a low affinity to 1-HO-didehydrolycopene as a substrate leading to the accumulation of large amounts of this carotenoid (Table 2). The small portion of 1,1′-$(HO)_2$-didehydrolycopene formed is then completely meta-bolized to 1,1′-$(HO)_2$-tetradehydrolycopene. In contrast, CrtC from *Rvi. gelatinosus* very efficiently synthesized 1,1′-$(HO)_2$-didehy-

drolycopene leading to its accumulation and to the formation of comparably high amounts of 1,1′-$(HO)_2$-tetradehydrolycopene (Fig. 1). Both carotenoids, 1,1′-$(HO)_2$-didehydrolycopene and 1,1′-$(HO)_2$-tetradehydrolycopene were postulated as intermediates in the biosynthetic pathway to spirilloxanthin in *Rvi. gelatinosus* (Steiger et al., 2000). From the carotenoid composition of *E. coli* expressing either $crtC_{Rc}$ or $crtC_{Rg}$, differential substrate specificities for both hydratases can be concluded. In vitro studies are in progress to analyze the enzyme kinetics of the *crtC* gene products from *Rvi. gelatinosus* and *Rba. sphaeroides*.

Acknowledgements

The authors want to thank Dr C. Astier, Centre National de la Recherche Scientific, Gif sur Yvette, France for providing plasmid pSO50 and pSO53 with the *crtC* and *crtD* genes from *Rubrivivax gelatinosus*.

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