Combinatorial Biosynthesis of Carotenoids in a Heterologous Host: A Powerful Approach for the Biosynthesis of Novel Structures

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Carotenoids are commercially important pigments with high antioxidative potential. A variety of structures can be biosynthesized in the heterologous host Escherichia coli *after transformation with combinations of genes from prokaryotes, lower and higher plants. Among the produced carotenoids are novel structures with superior antioxidative activity. In this article, the concept of the combinatorial biosynthesis approach,* E. coli *as a carotenoid production system, and metabolic engineering of precursor supply are covered.*

KEYWORDS:

antioxidants · carotenoids · carotogenic genes · metabolic engineering · terpenoids

1. Introduction

The biosynthesis of metabolites involves reaction sequences catalyzed by individual enzymes.[1] Specific genes encode these enzymes. Once the genes or complementary DNAs (cDNAs) of a pathway are known and available, they can be applied to establish this pathway in a heterologous host. In the case of carotenoids, this strategy has been used successfully to produce rare and even novel structures which have not been previously been detected in biological material or chemically synthesized.[2]

In microorganisms and plants where carotenoids are synthesized, their major function is protection against oxidative damage. These pigments are capable of quenching photosensitizers, interacting with singlet oxygen,[3] and scavenging peroxy radicals.[4] For humans, carotenoids with unsubstituted β-ionone end groups are precursors of vitamin A. Furthermore, evidence is accumulating that carotenoids play an important role in the prevention of degenerative diseases and cancer.[5] Several authors have suggested that the antioxidative activity of carotenoids may be an important factor for the role of carotenoids in human health.[6, 7] However, very little is known about the relationship between pharmaceutical potential, the structure of a certain carotenoid, and its antioxidative activity. Although hundreds of carotenoids with diverse chemical structures have been identified, only a very few are available for studies to evaluate their pharmaceutical potential. Some can be extracted from natural sources, but in the case of β-carotene (β,β-carotene), astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), and lycopene (Ψ,Ψ-carotene) the commercial demand for carotenoids is met by chemical synthesis. One possibility for overcoming the limited availability of many other carotenoids is the heterologous expression of carotenoid genes in suitable microorganisms. The noncarotenogenic yeasts *Candida utilis* and *Saccharomyces cerevisiae*[8] and especially the bacterium *Escherichia coli*[2] have been used for the synthesis of rare carotenoids. The following sections focus on the production of rare carotenoids in the heterologous host *E. coli* and describe how a limitation of carotenoid production caused by the supply of precursors can be alleviated by metabolic engineering of the early terpenoid pathway.[9]

2. Typical Structures of Carotenoids in Bacteria, Fungi, and Plants

Most carotenoids are C_{40} terpenoids differing in the number of conjugated double bonds, the structure of the end groups, and the oxygen-containing substituents. In certain groups of bacteria C_{30}, C_{45}, and C_{50} carotenoids can also be found.[10] To date, more than 600 naturally occurring carotenoids have been identified. They include intermediates and end products of different branches of biosynthetic pathways. Typical carotenoids for bacteria, fungi, and plants are shown in Scheme 1. Zeaxanthin (β,β-carotene-3,3′-diol) which is wide-spread in many organisms can be modified, especially in extremophile bacteria like *Thermus thermophilus*, to glucoside fatty acid esters.[11] Acyclic carotenoids like spheroidene (1-methoxy-3,4-didehydro-1,2,7′,8′-tetrahydro-Ψ,Ψ-carotene) and its 2-keto derivative are restricted to photosynthetic bacteria. In fungi, monocyclic carotenoids like plectaniaxanthin (3′,4′-didehydro-1′,2′-dihydro-β,Ψ-carotene-1′,2′-diol) are abundant.[12] In higher plants and green algae, lutein (β,ε-carotene-3,3′-diol) is the dominating carotenoid in the photosynthetic apparatus. This carotenoid contains a combination of a β- and an ε-ionone end group. Another typical plant carotenoid is antheraxanthin (5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol) with a 5,6-epoxy substituent.[10]

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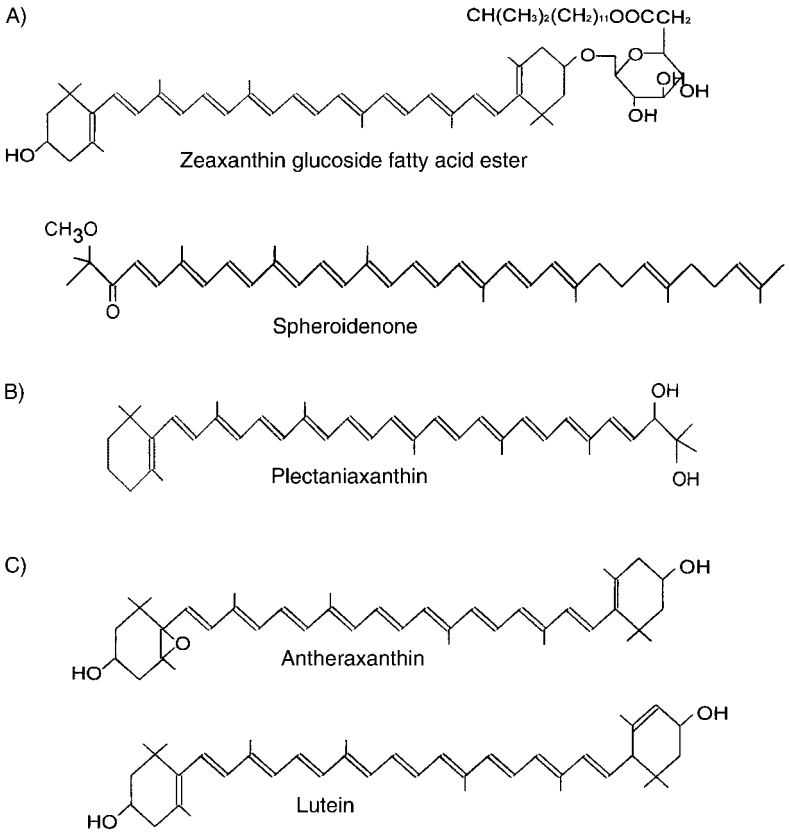


Scheme 1. *Typical carotenoid structures found in A) bacteria, B) fungi, and C) plants.*

3. Knowledge and Tools

For the heterologous biosynthesis of individual carotenoids the reaction sequences of the pathway have to be established, all genes involved must be available, and the catalytic properties of the encoded enzymes should have been elucidated. There is one case of a bifunctional gene and corresponding enzyme in which the expressed protein catalyzes two distinct and mechanistically unrelated carotenogenic reactions. The *crtBY* gene from *Xanthophyllomyces dendrorhous* encodes a protein involved in phytoene synthesis from geranylgeranyl pyrophosphate as well as in cyclization of lycopene into β-carotene.[13] Furthermore, in some cases, it can be very important to know to what extent an enzyme is specific for its genuine substrate. When other carotenoids in the pathway can also be converted, unwanted side reactions should be expected. On the other hand, a broad substrate specificity may qualify an enzyme for the synthesis of a special, unusual carotenoid.

3.1. Reaction sequences

All C_{40} carotenoids are derived from C_{20} geranylgeranyl pyrophosphate which is dimerized into phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-Ψ,Ψ-carotene) (Scheme 2 A). In a very similar reaction, two molecules of C_{15} farnesyl pyrophosphate are condensed to form C_{30} diapophytoene (7,8,11,12,7′,8′,11′,12′-octahydro-4,4′-diapocarotene). Subsequently, several double bonds are inserted into phytoene and diapophytoene. Phytoene desaturases exist which differ by the end product of the desaturation reaction. These products possess at least two and maximally five additional double bonds. In cyanobacteria, algae, and plants the reaction catalyzed by Pds/CrtP yields ζ-carotene (7,8,7′,8′-tetrahydro-Ψ,Ψ-carotene). Since the accumulating end carotenoids in these organisms are cyclic products of lycopene, another desaturase is essential for the conversion of ζ-carotene into lycopene. In Scheme 2 carotenoids are shown in their all-*trans* configuration. However, the reaction product of the plant-type ζ-carotene desaturase CrtQb preferentially forms 7,7′,9,9′-*cis* lycopene. Since this isomer cannot be cyclized to ionone end groups, isomerization to the all-*trans* form catalyzed by a specific carotene isomerase CrtH is essential.[14, 15] All types of CrtI enzymes including Al-1, the ζ-carotene desaturase crtQa, and also the isomerase CrtH share high homologies among each other. They are structurally completely unrelated to phytoene desaturase CrtP/Pds and ζ-carotene desaturase CrtQb/Zds, which form another family of proteins.[16]

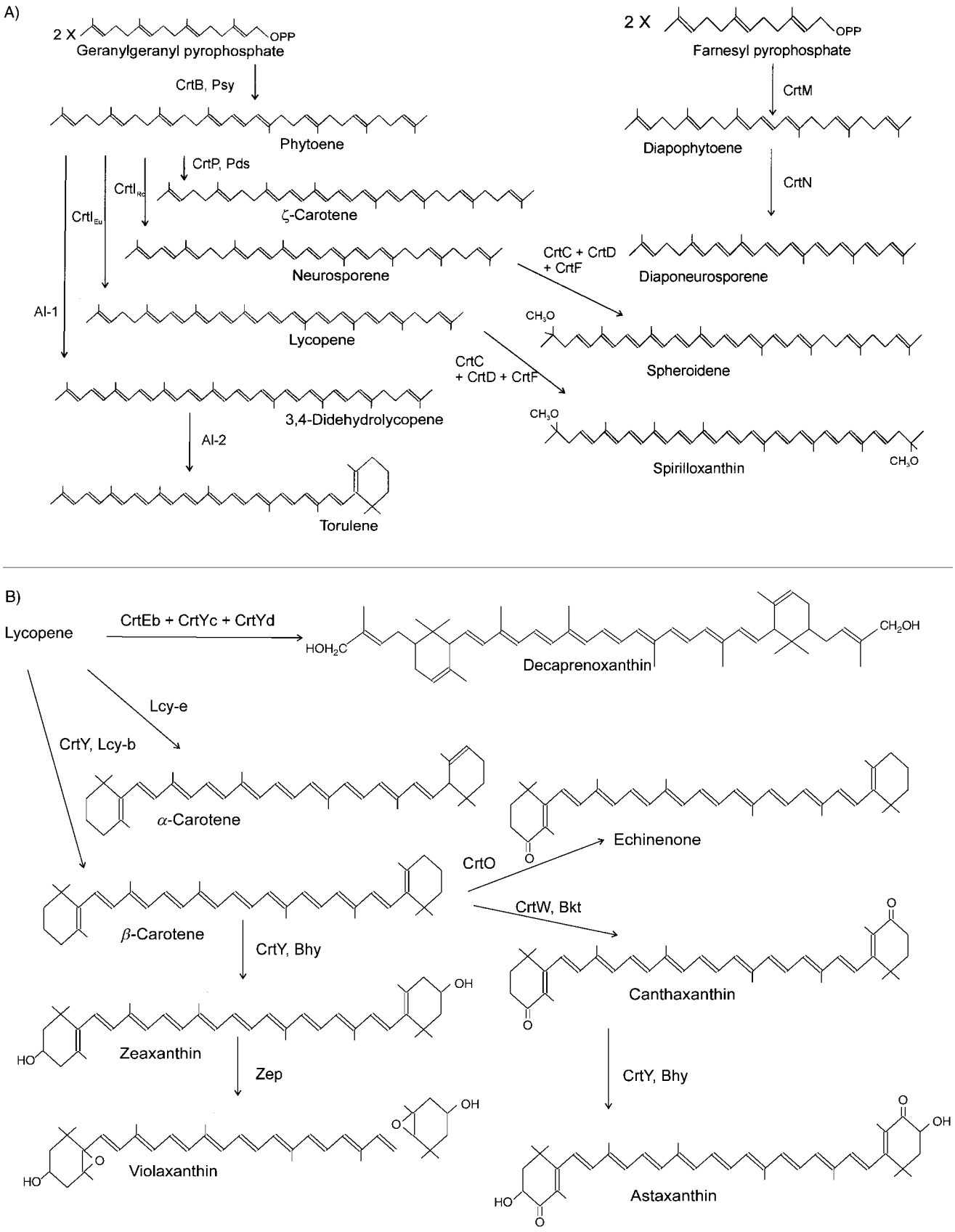
From neurosporene (7,8-dihydro-Ψ,Ψ-carotene) and lycopene, respectively, acyclic spheroidene and spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-Ψ,Ψ-carotene) are derived by the reaction of a 1,2-hydratase CrtC, a 3,4-desaturase CrtD, and an *O*-methylase CrtF.[17] Lycopene is also the substrate for chain elongation, leading to C_{50} decaprenoxanthin (2,2′-bis(4-hydroxy-3-methyl-2-butenyl)-ε,ε-carotene),[18] and for cyclization to α-carotene and β-carotene (Scheme 2 B). The latter carotene is hydroxylated at positions 3 and 3′ to form zeaxanthin and modified further to the 5,6,5′,6′-diepoxide violaxanthin (5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol). β-Carotene can also be converted into the 4-keto derivative echinenone (β,β-carotene-4-one) and the symmetric diketone canthaxanthin (β,β-carotene-4,4′-dione). 3,4-Didehydrolycopene which is the final product of the reactions catalyzed by the fungal Al-1 desaturase can be cyclized to torulene (3′,4′-didehydro-β,Ψ-carotene).[11]

3.2. Carotenogenic genes

All genes or cDNAs for the reactions outlined in Scheme 2 have been cloned and applied to carotenoid biosynthesis in heterologous hosts. The reactions catalyzed by the encoded enzymes are indicated in Table 1 with the converted substrates and the final products.

3.3. Substrate and product specificity of carotenogenic enzymes

In general, enzymes exhibit a high degree of specificity for their substrates. However when larger molecules like carotenoids are converted, the enzyme may not need to recognize the entire



Scheme 2. *Pathways leading to the heterologous formation of A) acyclic carotenoids and torulene, or B) cyclic products of lycopene in* E. coli.

Table 1. Carotenogenic genes and cDNAs for carotenoid biosynthesis in A) E. coli and B) metabolic engineering of the pathway.

Enzyme and/or gene	Substrate	Reaction product
A)		
C_{30} chain:		
diapophytoene synthase/*crtM*	FPP[a]	diapophytoene[20]
diapophytoene desaturase/*crtN*	diapophytoene	diaponeurosporene[20]
C_{40} chain:		
GGPP[a] synthase/*crtE*	FPP[a]	GGPP[a][35]
phytoene synthase/*crtB*	GGPP[a]	phytoene[35]
desaturases/*pds*	phytoene	poly-*cis* ζ-carotene[36]
$crtI_{Rc}$	phytoene	neurosporene[36]
$crtI_{Eu}$	phytoene	all-*trans* lycopene[36]
al-1	phytoene	3,4-didehydrolycopene[37]
crtQa	ζ-carotene	all-*trans* lycopene[38]
crtQb	ζ-carotene	poly-*cis* lycopene[14, 15]
crtH	poly-*cis* lycopene	all-*trans* lycopene[14, 15]
crtD	hydroxyneurosporene	demethylspheroidene[39]
lycopene β-cyclase/*crtY*	lycopene	β-carotene[25]
lycopene ε-cyclase/*lcy-e*	lycopene	δ-carotene[40]
hydroxylase/*crtZ*	β-carotene	zeaxanthin[28]
epoxydase/*zep*	zeaxanthin	violaxanthin (unpublished results)
ketolases/*crtA*	spheroidene	spheroidenone (unpublished results)
crtW, *bkt*	β-carotene	canthaxanthin[41]
crtO	β-carotene	echinenone[30]
hydratase/*crtC*	neurosporene	1-hydroxyneurosporene[39]
glycosilase/*crtX*	zeaxanthin	zeaxanthin
diglucoside[24, 25]		
$C_{45/50}$ chain:		
lycopene elongase/*crtEb*	lycopene	flavuxanthin[18]
		nonaflavuxanthin[18]
decaprenoxanthin synthase/ *crtYe-crtYf*	flavuxanthin	decaprenoxanthin[18]
B)		
1-deoxyxylulose-5-P synthase/*dxs*	glyceraldehyde/pyruvate	1-deoxyxylulose-5-P[9]
1-deoxyxylulose-5-P reductoisomerase/*dxr*	1-deoxyxylulose-5-P	2-C-methyl-D-erythritol-4-P[9]
isopentenyl pyrophosphate isomerase/*idi*	isopentenyl pyrophosphate	dimethylallyl pyrophosphate[9]
phosphoenolpyruvate synthase/*pps*	pyruvate	phosphoenolpyruvate[27]

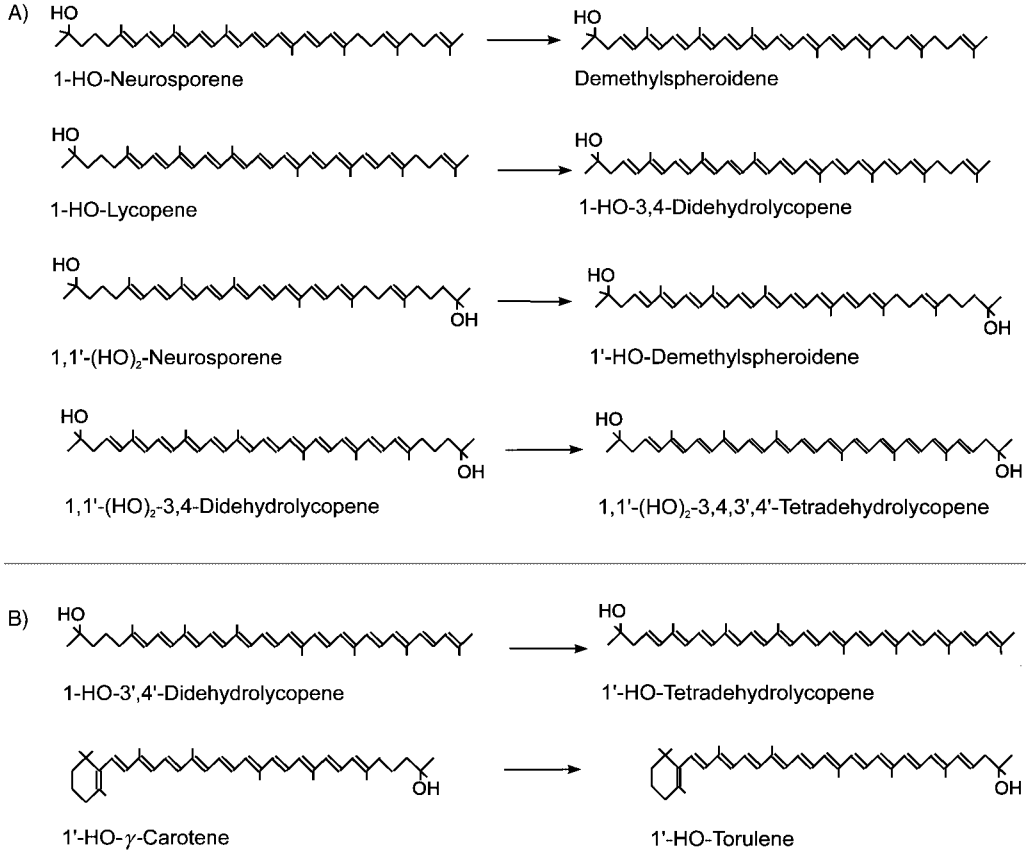
[a] Abbreviations: FPP = farnesyl pyrophosphate, GGPP = geranylgeranyl pyrophosphate.

substrate molecule but only a certain region of the molecule which is suitable for conversion.

Qualitative and quantitative substrate conversion studies have been carried out in vitro with carotenogenic enzymes. The most convenient way to prepare carotenogenic enzymes for functional characterization is by their overexpression in a heterologous host.[19] Although these enzymes are all membrane-associated or integrated into membranes, *E. coli* transformants carrying a plasmid with carotenogenic genes can be grown in such a way that high-pressure cell breaking results in a soluble and enzymatically active form. When the coding region of the gene is extended by an affinity group, for example, a 6 × His tag, purification of the expressed enzymes is highly facilitated. Many carotenogenic enzymes have been expressed, purified, and biochemically characterized by this way. Among the enzymes studied, several cases for a region-limited substrate specificity as pointed out above were found. For example, enzymes of the C_{30} carotenoid pathway can convert C_{40} substrates and vice versa.[20] A broad substrate specificity was also shown for CrtD, the 3,4-carotene desaturase from photosynthetic bacteria involved in the synthesis of spheroidene and spirilloxanthin (Scheme 2 A). In *Rhodobacter* and *Rubrivivax* species 1-HO-neurosporene, 1-HO-lycopene, and 1-CH_3O-1′-HO-3,4-didehydrolycopene all are substrates for this enzyme.[21, 22] But other related substrates are also efficiently converted (Scheme 3 A). Nevertheless, both highly homologous enzymes differ in their capability to convert 1-HO-3′,4′-didehydrolycopene into 1-HO-3,4,3′,4′-tetradehydrolycopene and 1′-HO-γ-carotene (1′-hydroxy-1′,2′-dihydro-β,Ψ-carotene) into 1′-HO-torulene (1′-hydroxy-1′,2′-dihydro-3′,4′-didehydro-β,Ψ-carotene; Scheme 3 B). Only the enzyme from *Rubrivivax* can catalyze both reactions in substantial rates.

4. *Escherichia coli* as a Heterologous Host for Carotenoid Biosynthesis

Escherichia coli was the first heterologous host in which carotenoids were produced. In the course of cloning of a gene cluster from *Erwinia herbicola* yellow pigmentation of *E. coli* cells was observed.[23] Latter analysis of transformants carrying this and a similar gene cluster from *Erwinia uredovora* demonstrated the existence of a carotenogenic pathway to zeaxanthin glucoside.[24, 25]



Scheme 3. *Reactions carried out in vitro by the product of the* crtD *gene from A) both* Rhodobacter spheroides *and* Rubrivivax gelatinosous *or B) exclusively by the enzyme from* Rubrivivax gelatinosous.

4.1. The production system *E. coli*

E. coli is a very convenient host for heterologous carotenoid production. Most of the carotenogenic genes from bacteria, fungi, and higher plants can be functionally expressed in this bacterium. Furthermore, plasmids belonging to different incompatibility groups with different antibiotic resistance markers are available. They can all be introduced simultaneously in *E. coli* for carotenoid synthesis allowing combinations of individual genes. The potential of *E. coli* as a carotenoid production system has been reviewed recently.[2] Table 2 comprises the individual carotenoids which can be synthesized in *E. coli*. They include carotenoids of different chain lengths, cyclic and acyclic structures with shorter or longer conjugated double bond systems, unsubstituted hydrocarbons, or hydroxy, keto, and epoxy derivatives.

Table 2. *Gene combinations and production of carotenoids in* E. coli[a]

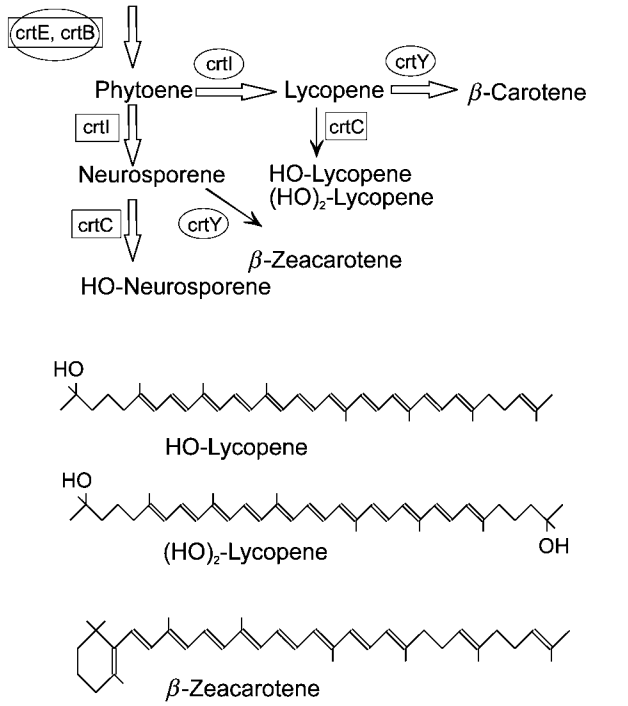
Erwinia crtE, *crtB*, *crtI*$_{Eu}$, *crtY*, *crtX*[25, 35]	phytoene, lycopene, ζ-carotene, zeaxanthin, zeaxanthin glucoside
+ *Rhodobacter crtI*$_{Rc}$ + *Erwinia crtY*[42]	**7,8-dihydro-β-carotene**,[b] **7,8,7′,8′-tetrahydro-β-carotene**[b]
+ *Rhodobacter/Rubrivivax crtI*$_{Rc}$, *crtD*, *crtC*[26, 39, 42]	1-HO-lycopene, 1,1′-(HO)$_2$-lycopene, 1′-HO-γ-carotene, 1-HO-neurosporene, demethylspheroidene, **1′,3-(HO)$_2$-γ-carotene**,[b] **1-HO-3,4-didehydrolycopene**,[b] **1,1′-(HO)$_2$ – 3,4-didehydrolycopene**,[b] **1,1′-(HO)$_2$-3,4,3′,4′-tetradehydrolycopene**,[b] **7,8-dihydrozeaxanthin**, **3-HO-β-zeacarotin**
+ *Neurospora al-1* + *Rhodobacter/Rubrivivax crtC*, *crtD*[26]	3,4-didehydrolycopene, **1-HO-3′,4′-didehydrolycopene**,[b] **1-HO-3,4,3′,4′-tetradehydrolycopene**[b]
+ *Erwinia crtI*$_{Eu}$, *crtY* + *Synechocystis crtO*[30]	echinenone
+ *Erwinia crtI*$_{Eu}$, *crtY* + *Haematococcus bkt*[43]	canthaxanthin
+ *Synechococcus crtP*[36]	ζ-carotene
+ *Erwinia crtI*$_{Eu}$ + *Corynebacterium crtEb*[18]	flavuxanthin, nonaflavuxanthin
Staphylococcus crtM[20]	diapophytoene
+ *Staphylococcus crtN*[20]	diapo-ζ-carotene, diaponeurosporene
+ *Staphylococcus crtN* + *Anabaena crtQa*[20]	diapolycopene

[a] Carotenoids in bold face have not been found before in biological material. [b] These compounds were identified by NMR spectroscopy; other compounds were identified by HPLC, optical methods, and mass spectroscopy.

4.2. Reactions and application of combinatorial carotenoid biosynthesis

A combination of genes from different organisms which follow different branches of a pathway makes it possible to synthesize novel compounds. This strategy only works, however, when the recognition of the substrate by the gene product is restricted to a certain region of the carotenoid structure. An example is given in Scheme 4. The gene products marked by circles originate from *Erwinia uredovora*, a bacterium which synthesizes zeaxanthin diglucoside via β-carotene. Those in boxes are from the *Rhodobacter* pathway to spheroidene via 1-HO-neurosporene. The genes *crtE* and *crtB* are common to both organisms. By combining *crtI* from *Rhodobacter* with *crtY* from *Erwinia* in a background of *crtB* and *crtE*, β-zeacarotene (7′,8′-didehydro-β,Ψ-carotene) is formed. Alternatively, a combination of *crtI* from *Erwinia* and *crtC* from *Rhodobacter* results in the biosynthesis of 1-HO-lycopene and 1,1′-$(HO)_2$-lycopene. These three carotenoids do not exist in the carotenogenic pathways of *Rhodobacter* and *Erwinia*.

A combinatorial approach as explained here resulted in the formation of numerous novel carotenoids depending on the available genes. In Table 2 novel carotenoids are indicated in bold face. They include 1-hydroxylated acyclic structures with up to 13 conjugated double bonds.[26] Their antioxidative potential was superior to other related carotenoids.

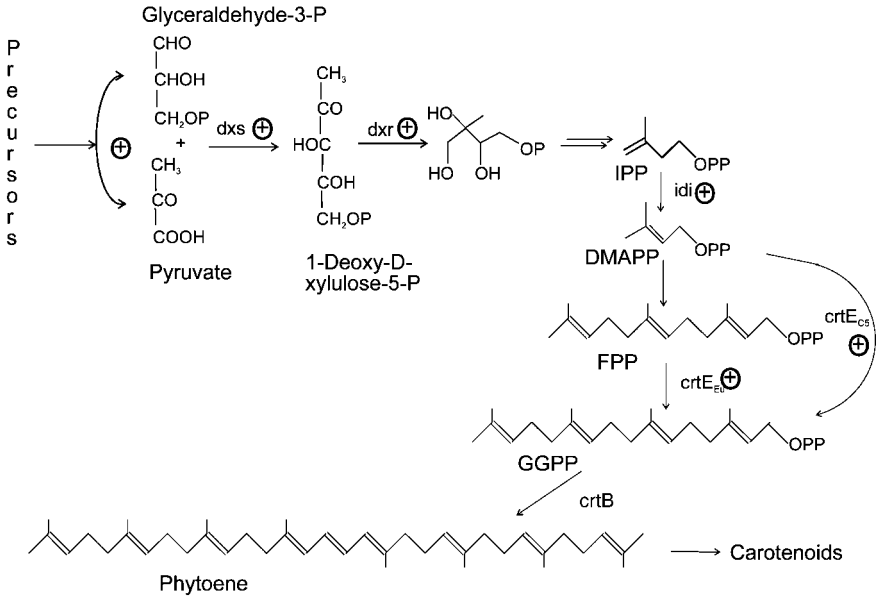


Scheme 4. *Principle of combinatorial biosynthesis of novel carotenoids. Gene products in circles originate from* Erwinia uredovora, *gene products in boxes from* Rhodobacter capsulatus; *crtE and crtB are common to both species.*

4.3. Metabolic engineering of precursor supply

E. coli is a non-carotenogenic bacterium. All precursors for carotenoid formation in the transformants with *crt* genes must be diverted from other biosynthetic pathways. This leads to a limitation of carotenoid yields. The problem of precursor supply could be overcome by metabolic engineering of its deoxyxylulose 5-phosphate pathway which provides the prenyl pyrophosphates for carotenoid synthesis. As indicated in Scheme 5, transformation with the genes for overexpression of 1-deoxy-D-xylulose 5-phosphate synthase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase, and isopentenyl pyrophosphate stimulated carotenogenesis.[9] Another important factor for carotenoid biosynthesis in *E. coli* is the balance between the pools of pyruvate and glyceraldehyde 5-phosphate.[27] By overexpressing the gene which encodes phosphoenolpyruvate synthase or inactivation of the gene encoding pyruvate kinase, carotenoid formation was enhanced; this indicates that formation of glyceraldehyde 3-phosphate has to be increased versus pyruvate, because these are both simultaneous substrates of 1-deoxy-D-xylulose 5-phosphate synthase (Scheme 5).

For geranylgeranyl pyrophosphate synthase which is the final limiting enzyme in carotenoid synthesis it was demonstrated that the expression levels are important.[28] By establishing a regulatory circuit that controls the expression of foreign carotenogenic genes, carotenoid production was substantially increased.[29]



Scheme 5. *Limiting reactions in the early pathway of* E. coli *leading to the formation of prenyl pyrophosphates. Overexpression of enzymes of the reactions indicated by ⊕ resulted in increased formation of carotenoids. IPP = isopentenyl pyrophosphate, DMAPP = dimethylallyl pyrophosphate, FPP = farnesyl pyrophosphate, GGPP = geranylgeranyl pyrophosphate.*

5. Conclusion and Perspectives

The major importance of *E. coli* as a carotenoid production system is to provide a broad selection of carotenoid structures. The structurally diverse carotenoids may be useful to study the relationship between structural features and antioxidative activities as well as pharmaceutical properties. When the first substantial data are available, predictions can be made about how structures with increased potential may look. Then, the combinatorial approach will allow, at least to a certain extent, desired carotenoid molecules to be tailored.

As outlined in this review, many diverse carotenoid structures can be heterologously produced in transgenic *E. coli*. One of the few limitations with respect to carotenogenic reactions is the modification of the ε-ionone ring, since the gene for its hydroxylation has not been cloned yet. Most of the characterized carotenogenic enzymes modify their substrates symmetrically at both ends of the molecule. Only a few asymmetrically acting enzymes, like a β-carotene monoketolase[30] or a lycopene monocyclase (unpublished results), are known and their genes cloned. Others may be cloned from special organisms. However, an alternative way may be the mutagenesis of genes for symmetrical enzymes and selection for asymmetric carotenogenesis or molecular breeding. Random mutagenesis of geranylgeranyl pyrophosphate synthase has already been successful in increasing carotenoid production in *E. coli*.[31] Application of gene shuffling resulted in the modification of substrate and/or product specificity of carotenogenic enzymes.[32, 33] Phytoene desaturase genes from two *Erwinia* species were shuffled randomly and a recombined gene encoding an enzyme which introduced six instead of four double bonds was obtained. In a similar way a lycopene cyclase with several amino acid exchanges was created which was able to convert 3,4-didehydrolycopene into torulene.[33] By gene shuffling, phytoene desaturase could also be modified to synthesize ζ-carotene which is a nonaccumulating intermediate of the four-step process in the wild-type enzyme. Another interesting approach leading to the accumulation of asymmetrical intermediates of the phytoene desaturase and lycopene cyclase reaction was described recently.[34] The phytoene desaturase and lycopene cyclase genes were cloned into various plasmids together with mRNA stability control elements which modulated the expressed enzyme levels. In some of the *E. coli* transformants ζ-carotene accumulated together with neurosporene and γ-carotene; a fact indicating that an alteration of the balance and levels of enzymes in the pathway can lead to the accumulation of intermediates of the carotenogenic pathway.

Metabolic engineering of the *E. coli* terpenoid biosynthetic reactions led to a considerable increase of carotenoid production. All the lipophilic carotenoids are sequestered in the cell membranes. Thus, first indications arise that deposition of carotenoids becomes a limiting factor in *E. coli*.[9] Therefore, future activities may aim to extend the carotenoid storage capacity by genetic modification of the amount of *E. coli* membranes or by establishing plastoglobuli-like structures.

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