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MINI-REVIEW

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Metabolic engineering towards biotechnological production of carotenoids in microorganisms

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Abstract Carotenoids are important natural pigments produced by many microorganisms and plants. Traditionally, carotenoids have been used in the feed, food and nutraceutical industries. The recent discoveries of health-related beneficial properties attributed to carotenoids have spurred great interest in the production of structurally diverse carotenoids for pharmaceutical applications. The availability of a considerable number of microbial and plant carotenoid genes that can be functionally expressed in heterologous hosts has opened ways for the production of diverse carotenoid compounds in heterologous systems. In this review, we will describe the recent progress made in metabolic engineering of non-carotenogenic microorganisms for improved carotenoid productivity. In addition, we will discuss the application of combinatorial and evolutionary strategies to carotenoid pathway engineering to broaden the diversity of carotenoid structures synthesized in recombinant hosts.

Introduction

Carotenoids form an important medicinal and biotechnological class of natural pigments produced by many microorganisms and plants. These structurally diverse pigments have many different biological functions, such as species-specific coloration, photo protection, and light harvesting, and they serve as precursors for many hormones (Vershinin 1999). Carotenoids are used commercially as food colorants, animal feed supplements and, more recently, as nutraceuticals for cosmetic and pharmaceutical purposes. Presently however, only a few carotenoids (β-carotene, lycopene, astaxanthin, canthaxanthin, capsanthin, lutein, annatto, β-apo-8-carotenal, β-apo-8-carotenal-ester) can be produced commercially by chemical synthesis, fermentation or isolation from the small number of abundant natural sources (Johnson and Schroeder 1995). The 1999 world market for carotenoids was US$ 750–800 million and projections estimate around US$ 1 billion in 2005 (Business Communications Co. 2000). The demand and market for carotenoids is anticipated to change drastically with the discovery that carotenoids exhibit significant anti-carcinogenic activities and play an important role in the prevention of chronic diseases (Bertram 1999; Edge et al. 1997; Rock 1997; Singh and Lippman 1998; Smith 1998). In fact, carotenoids play an exceptional role in the fast-growing 'over the counter (OTC) medicine' and nutraceutical sector (Ulrich Merz 2000).

The increasing importance of carotenoids in the food, nutraceutical, feed and pharmaceutical markets has initiated renewed efforts to find ways of producing additional carotenoid structures in useful quantities. Because plants, algae and microorganisms synthesize hundreds of different complex chemical carotenoid structures (Britton 1998; Straub 1987), and a number of carotenoid biosynthetic pathways (albeit only a fraction of the total number) have been elucidated on a molecular level, metabolic engineering of microorganisms can provide a means towards economic production of carotenoid structures that are otherwise inaccessible.

This review describes progress in engineering non-carotenogenic microorganisms for (1) the production of carotenoids and (2) the synthesis of new carotenoid structures by using combinatorial and in vitro evolutionary strategies.

Biosynthesis of carotenoids

Biochemical analysis of carotenoid biosynthesis, molecular genetics, and more recently genomics has resulted in the elucidation of the main routes of synthesis of acyclic and cyclic carotenoids at a molecular level (biosynthesis and molecular biology of carotenoids is comprehensively reviewed in Britton 1998). More than 150 genes encod-

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Table 1 Selection of genes encoding carotenoid biosynthetic enzymes

Enzyme	Gene	Organism	Accession number
Formation of carotenoid backbone			
Dehydrosqualene synthase[a]	*crtM*	*Staphylococcus aureus*	X73889
GGPP synthase	*crtE*	*Erwinia uredevora*	D90087
	crtE	*Synechocystis* PCC6803	D90899
	al-3	*Neurospora crassa*	X53979
	gps	*Arabidopsis thaliana*	L25813
Phytoene synthase	*crtB*	*Agrobacterium aurantiacum*	D58420
	crtB	*Synechocystis* PCC6803	X69172
	crtB	*Brevibacterium linens*	T51118
	al-2	*Neurospora crassa*	L27652
	psy	*Arabidopsis thaliana*	L25812
Formation of acyclic carotenoids and their derivatives			
Dehydrosqualene desaturase[a]	*crtN*	*Staphylococcus aureus*	X73889
Phytoene desaturase			
Two desaturations	*crtP*	*Synechocystis* PCC6803	X62574
	pds1	*Arabidopsis thaliana*	L16237
Three desaturations	*crtI*	*Rhodobacter capsulatus*	Z11165
Four desaturations	*crtI*	*Erwinia uredevora*	D90087
Up to five desaturations	*al-1*	*Neurospora crassa*	M57465
ζ-Carotene desaturase	*crtQ*	*Synechocystis* PCC6803	X62574
	zds	*Capsicum annuum*	X68058
Hydroxyneurosporene synthase	*crtC*	*Rhodobacter sphaeroides*	X82458
	crtC	*Rubrivivax gelatum*	U73944
Methoxyneurosporene desaturase	*crtD*	*Rhodobacter capsulatus*	Z11165
	crtD	*Rubrivivax gelatum*	U73944
Hydroxyneurosporene-O-methyltransferase	*crtF*	*Rhodobacter sphaeroides*	X82458
Spheroidene monooxygenase	*crtA*	*Rhodobacter capsulatus*	Z11165
Lycopene elongase[b]	*crtEb*	*Corynebacterium glutamicum*	AF159510
Formation of cyclic carotenoids and their derivatives			
Lycopene-β-cyclase	*crtY*	*Erwinia uredevora*	D90087
	crtY	*Synechocystis* PCC6803	X74599
	crtL-b	*Arabidopsis thaliana*	Z29211
Lycopene-ε-cyclase	*crtL-e*	*Arabidopsis thaliana*	U50738
β-Carotene hydroxylase	*crtZ*	*Agrobacterium aurantiacum*	D58420
	crtR-b1	*Capsicum annuum*	Y09225
Zeaxanthin glucosylase	*crtX*	*Erwinia herbicola* EhoI	M87280
β-Carotene C(4) oxygenase	*crtW*	*Agrobacterium aurantiacum*	D58420
	crtO	*Synechocystis* PCC6803	D64004
	crtW	*Alcaligenes* PC1	D58422
	crtO/bkt	*Haematococcus pluvialis*	X86782/D45881
Zeaxanthin epoxidase	*zep1*	*Arabidopsis thaliana*	T45502
Violaxanthin deepoxidase	*vde1*	*Arabidopsis thaliana*	N37612
Violaxanthin cleavage	*vp14*	*Zea mays*	U95953
Capsanthin/capsorubin synthase	*ccs*	*Capsicum annuum*	X77289
β-Carotene desaturase	*crtU*	*Streptomyces griseus*	X95596
	crtU	*Brevibacterium linen*	AAF65586
Decaprenoxanthin synthase[b]	*crtYe/Yf*	*Corynebacterium glutamicum*	AF159510

[a] C30 carotenoid biosynthesis; [b] C50 carotenoid biosynthesis

ing 27 different carotenoid (crt) enzymes have been cloned from bacteria, plants, algae and fungi (Table 1). With few exceptions, carotenoid genes are functionally expressed in different heterologous hosts and enzymes from distant phylogenetic species can assemble into a functional membrane-bound multi-enzyme complex at which carotenoid biosynthesis presumably takes place (Britton 1998). However, little is known about the biosynthesis of carotenoids containing additional modifications of the end groups – the polyene chain and the methyl groups – or the molecular rearrangements that contribute to the tremendous structural diversity of carotenoids. At present, hundreds of individual carotenoids have been characterized (Straub 1987) and novel carotenoids continue to be isolated.

Isoprenoid pathway

All carotenoids are derived from the isoprenoid (or terpenoid) pathway. Two pathways, the mevalonate and the non-mevalonate (or pyruvate/glyceraldehyde-3-phos-

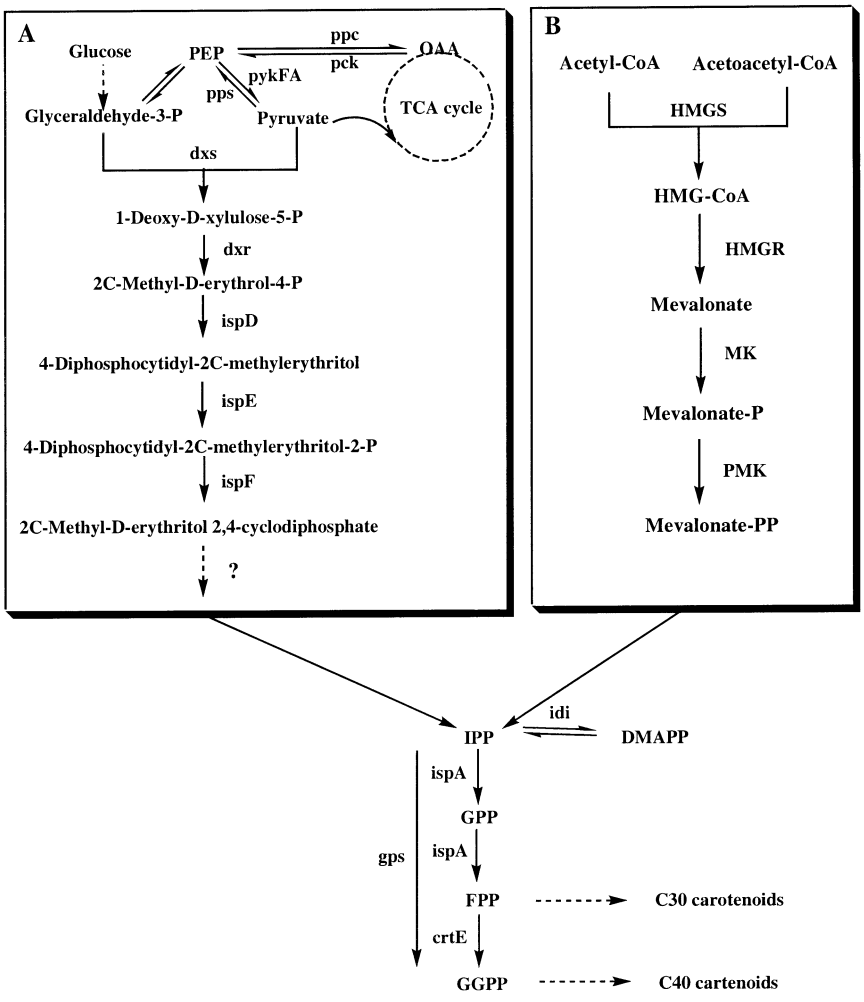

Fig. 1 Biosynthesis of isoprenoids by the non-mevalonate (**A**) and mevalonate (**B**) pathway. *dxs* 1-deoxy-D-xylose 5-phosphate synthase, *dxr* 1-deoxy-D-xylose 5-phosphate reductase, *ispD* 4-diphosphocytidyl-2C-methyl erythritol synthase, *ispE* 4-diphosphocytidyl-2C-methyl-D-erythritol kinase, *ispF* 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *ispA* geranylgeranyl pyrophosphate synthase, *idi* isopentenyl diphosphate isomerase, *pck* phosphoenolpyruvate carboxykinase, *ppc* phosphoenolpyruvate carboxylase, *pykFA* pyruvate kinase, *pps* phosphoenolpyruvate synthase, *gps* geranylgeranyl diphosphate synthase, *HMGS* HMG-CoA synthase, *HMGR* HMG-CoA reductase, MK mevalonate kinase, PMK phosphomevalonate kinase. Several enzymes involved in intermediate steps (indicated as a *question mark*) leading to IPP via the non-mevalonate pathway are still unknown. OAA Oxaloacetic acid, PEP phosphoenolpyruvate, TCA tricarboxylic acid

phate) pathways, lead to the formation of the first isoprene unit, isopentenyl pyrophosphate (IPP), in different species (Fig. 1). The non-mevalonate pathway has only recently been discovered (Rohmer 1999). Until this discovery, the mevalonate pathway was thought to be the only route to IPP. In archaea, fungi, animals and plants (except plastids), IPP is synthesized from mevalonic acid with acetyl-CoA as a starting precursor. In eubacteria (and plant plastids), IPP synthesis proceeds via an initial condensation reaction between pyruvate and glyceraldehyde-3-phosphate followed by several additional reaction steps, some of which are yet to be discovered (reviewed in Eisenreich et al. 2001). The discovery of the eubacterial non-mevalonate pathway has provided the means for engineering the isoprenoid flux in non-carotenogenic microorganisms to improve carotenoid productivity (discussed later).

After IPP has been synthesized by either of the two routes, the first committed step in the synthesis of the various terpenoids is chain elongation by successive head-to-tail condensation of dimethylallyl pyrophosphate (DMAPP), the reactive IPP isomer, initially to IPP and next to the growing polyprenyl pyrophosphate chain. Isomerization of IPP to DMAPP is catalyzed by isopentenyl pyrophosphate isomerase (*idi*). Chain elongation is catalyzed by prenyl transferases with different chain length specificities (reviewed in Ogura and Koyama 1998). The short-chain length prenyl transferases synthesize geranyl PP (GPP, C10), farnesyl PP (FPP, C15) or geranylgeranyl PP (GGPP, C20), which are the precursors of mono-, di-, and tri-terpenes and carotenoids. Medium and long-chain prenyl transferases synthesize the tails of quinones.

FPP (C15) and GGPP (C20) are the immediate precursors of C30 and C40 carotenoids. FPP is synthesized by an FPP synthase (*ispA* in *Escherichia coli*), whereas GGPP formation is catalyzed by a GGPP synthase (*crtE*, *al-3*) (Table 1). GGPP synthases with different affinities for allylic diphosphate substrates have been isolated from a number of organisms. For example, the *Erwinia uredevora* GGPP (*crtE*) converts FPP to GGPP, while the GGPP synthase (*gps*) from *Archaeoglobus fulgidus* produces GGPP directly from IPP (Wang et al. 1999).

Formation of the carotenoid backbone

Tail-to-tail condensation of two molecules of GGPP with elimination of two diphosphate groups is catalyzed by phytoene synthase (*crtB*, *al-2*) and yields the first color-

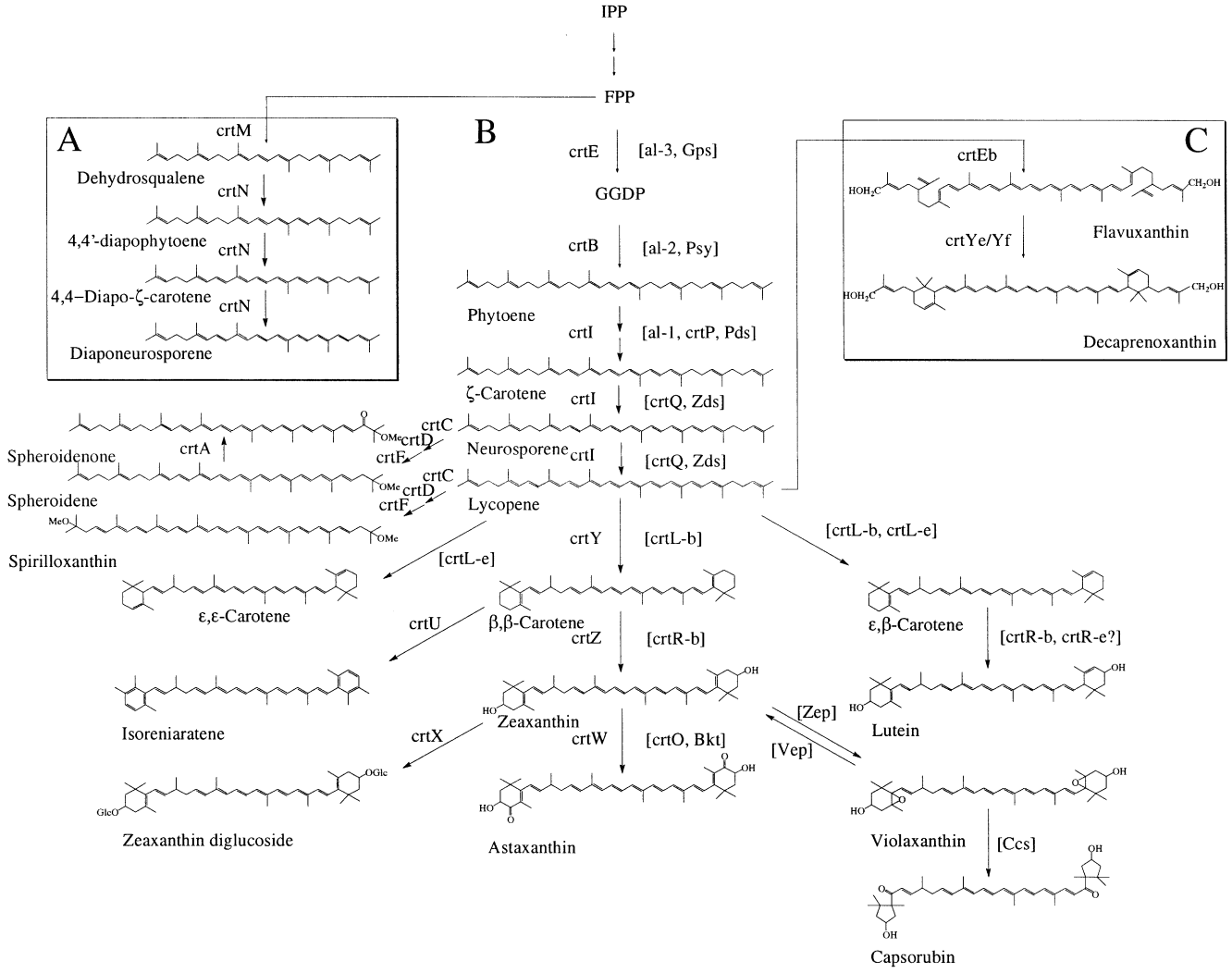

Fig. 2 Carotenoid biosynthesis pathways in microorganisms and plants. Biosynthesis pathways of C30 (**A**), C40 (**B**) and C50 (**C**) carotenoids in microorganisms and plants from which the enzymes have been cloned. Terminal products of biosynthesis routes are shown. Enzymes from plants, algae and cyanobacteria are given in parentheses. (**A**) *crtM* dehydrosqualene synthase, *crtN* dehydrosqualene desaturase. (**B**) *crtE, al-3, gps* GGPP synthase; *crtB, al-2, psy* phytoene synthase; *crtI, al–1, crtP, pds* phytoene desaturase; *crtQ, zds* ζ-carotene desaturase; *crtY, crtL-b* lycopene β-cyclase; *crtL-e* lycopene ε-cyclase; *crtZ, crtR-b* β-carotene hydroxylase; *crtW, crtO, bkt* β-carotene C(4) oxygenase; *crtX* zeaxanthin glucosylase; *zep* zeaxanthin epoxidase; *vep* violaxanthin deepoxidase; *ccs* capsanthin/capsorubin synthase; *crtU* β-carotene desaturase; *crtC* hydroxyneurosporene synthase; *crtD* methoxyneurosporene desaturase; *crtF* hydroxyneurosporene-O-methyltransferase; *crtA* spheroidene monooxygenase. (**C**) *crtEb* Lycopene elongase, *crtYe/Yf* heterodimeric decaprenoxanthin synthase

less C40 carotenoid phytoene. Similarly, dehydrosqualene synthase (*crtM*) from *Staphylococcus aureus* (Wieland et al. 1994) synthesizes the first colorless C30 carotenoid dehydrosqualene from two FPP molecules (Fig. 2). Subsequent desaturation reactions serve to increase the number of conjugated double bonds in phytoene or dehydrosqualene to produce colored carotenoids. Different phytoene desaturase genes have been cloned that introduce two, three, four or five double bonds into phytoene to produce ζ-carotene (plants, cyanobacteria, algae), neurosporene (*Rhodobacter*), lycopene (most eubacteria and fungi) or 3,4-didehydrolycopene (e.g., *Neurospora crassa*) (Bartley et al. 1999; Raisig et al. 1996; Ruiz-Hidalgo et al. 1997; Schmidhauser et al. 1990; Verdoes et al. 1999), respectively. A dehydrosqualene desaturase (*crtN*) from *S. aureus* (Wieland et al. 1994) introduces three double bonds into dehydrosqualene to form diaponeurosporene (Fig. 2).

Very recently, a novel gene encoding a prenyl transferase, lycopene elongase (*crtEb*) from *Corynebacterium glutamicum*, that catalyzes the condensation of one molecule DMAPP to either end of lycopene to produce the C50 carotenoid flavuxanthin has been isolated (Krubasik et al. 2001b) (Fig. 2).

Acyclic carotenoids

Presently, only genes encoding enzymes involved in acyclic xanthophyll biosynthesis from phototrophic bacteria have been cloned (Armstrong et al. 1989; Komori et al. 1998; Lang et al. 1995; Ouchane et al. 1997) (Fig. 2).

In purple bacteria, desaturation at C3,4, introduction of hydroxy and methoxy groups at C1(C1′) of neurosporene and lycopene and, under aerobic conditions, a keto-group at C2 of neurosporene lead to the synthesis of the acyclic xanthophylls spheroidene or spheroidenone and spirilloxanthin, respectively.

Cyclic carotenoids

Synthesis of cyclic carotenoids involves cyclization of one or both end groups of lycopene and three types of ionone rings can be formed. Typically, β-rings are introduced, but formation of ε-rings is common in higher plants, and a few carotenoids with γ-rings are also found, for example, in certain fungi (Fig. 2). Genes encoding lycopene-β-cyclases, catalyzing β-ring formation, have been cloned from a number of bacteria and plants. More recently, genes encoding lycopene-ε-cyclases have been isolated from plants (Cunningham et al. 1996; Matsumura et al. 1997). Whereas dicyclic products are formed by the β-lycopene cyclase, plant ε-lycopene cyclases usually synthesize monocyclic ε,ψ-carotene with the exception of lettuce ε-cyclase that forms ε,ε-carotene (Cunningham and Gantt 1998).

Most cyclic carotenoids contain at least one oxygen function at one of the ring carbon atoms. Cyclic carotenoids with keto-groups at C4(C4′) and/or hydroxy groups at C3(C3′) (e.g., zeaxanthin, astaxanthin, echinenone and lutein) are widespread in microorganisms and plants. To date, only genes encoding β-ring modifying enzymes have been cloned, including a number of β-carotene C(3) hydroxylases (Bouvier et al. 1998; Linden 1999) and β-carotene C(4) oxygenases (Fernandez-Gonzalez et al. 1997; Kajiwara et al. 1995; Lotan and Hirschberg 1995; Misawa et al. 1995a, b). The leaf xanthophylls violaxanthin and neoxanthin and the algae carotenoids such as fucoxanthin contain β-rings with a C(5,6)-epoxy group. The C(5,6)-epoxy-β-end group serves as a key intermediate in the formation of a variety of other unusual end groups, e.g., the cyclopentyl end group of capsanthin and capsorubin synthesized in *Capsicum annuum* (red pepper). In the past few years plant genes encoding zeaxanthin C(5,6) epoxidase and violaxanthin C(5,6) deepoxidase and pepper capsanthin/capsorubin synthase catalyzing κ-ring formation from the 3-hydroxy-5,6-epoxy-β-rings in violaxanthin and antheraxanthin have been identified (Cunningham and Gantt 1998).

Recent additions to the collection of *crt* genes are genes encoding β-carotene desaturases from *Streptomyces griseus* (Schumann et al. 1996) and *Brevibacterium linens* (Krugel et al. 1999) for isorenieratene (contains aromatic end groups) synthesis and a gene encoding decaprenoxanthin synthase from *Corynebacterium glutamicum* involved in decaprenoxanthin synthesis (Krubasik et al. 2001a) (Fig. 2).

Metabolic engineering of microbial carotenoid production

Most carotenoid genes and gene clusters have been cloned and expressed in the genetically well-established non-carotenogenic host *E. coli*. Carotenoid production levels in *E. coli* cells transformed with heterologous carotenoid genes, however, are typically low [~1 mg/g dry cell weight (DCW); see e.g., Ruther et al. 1997] compared to the up to several hundred-fold higher levels produced by carotenogenic algae and microbial strains such as *Dunalliella*, *Haematococcus*, *Flavobacterium*, and *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) (Johnson and Schroeder 1995). High-yield production of carotenoids in engineered microbial hosts will require (1) optimization of the available isoprenoid precursor pool, (2) balancing the expression of carotenogenic genes for efficient transformation of precursors to desired carotenoid compounds, and (3) provision of sufficient storage capabilities for the mostly lipophilic carotenoids either by engineering, for example, additional membrane storage or by selecting a suitable heterologous host with known high storage capabilities for hydrophobic compounds.

Optimization of the precursor pool

E. coli and other non-carotenogenic microbial cells have only a limited supply of isoprenoid precursors for the synthesis of metabolites like dolichols and quinones that are only needed by the cell in small quantities. Because carotenoid biosynthesis in engineered *E. coli* cells draws from the same limited precursor pool, increasing the isoprenoid flux should consequently translate into higher carotenoid production. Efforts have therefore been directed at overexpression of different enzymes of the non-mevalonate isoprenoid pathway (Fig. 1) in carotenoid-synthesizing *E. coli* cells.

Several studies showed that overexpression of 1-deoxy-D-xylose 5-phosphate synthase (*dxs*), which catalyzes the first step in the non-mevalonate pathway, improved lycopene production 2 to 3-fold (Harker and Bramley 1999; Matthews and Wurtzel 2000; Verdoes and van Ooyen 1999). Co-expression of *dxs* and 1-deoxy-D-xylose 5-phosphate reducto-isomerase (*dxr*), the enzyme next to DXS in the pathway, further improved lycopene yields 1.4 to 2-fold (Kim and Keasling 2001). An even larger effect on carotenoid production was observed when *idi* was overexpressed, indicating that the isomerization of IPP to DMAPP is indeed a rate-limiting step in isoprenoid biosynthesis. For example, Kajiwara et al. (1995) reported a 3.6 to 4.5-fold higher lycopene and a 2.7-fold higher β-carotene (~1.3 mg g DCW^{-1}) production when *idi* was overexpressed, co-expression of *idi* with either *dxs* or *dxr* increased zeaxanthin levels to 1.6 mg g DCW^{-1} (Albrecht et al. 1999), and astaxanthin production was 6-fold higher when *idi* was overexpressed (Wang et al. 1999).

To identify additional rate-controlling steps in isoprenoid and carotenoid biosynthesis, Wang et al. (1999) reconstructed the pathway leading from IPP to GGPP by converting IPP directly to GGPP by employing a multifunctional GGPP synthase (*gps*) from *A. fulgidus*. Overexpression of different gene combinations (*gps* alone; *gps* + *idi*; *crtE* alone; *crtE* + *isp*A, *crtE* + *ispA* + *idi*) in *E. coli* cells transformed with astaxanthin biosynthetic genes revealed that GGPP synthesis is the first rate-controlling step in carotenoid biosynthesis because it is relieved by overexpression of *crtE* or *gps*. The bottleneck then shifts to IPP isomerization, which is overcome by overexpression of *idi* in addition to *crtE*. The final rate-limiting step is synthesis of FPP, which is relieved by overexpression of *crtE*, *idi* and *ispA* resulting in a 12-fold increase of astaxanthin yield (390 μg g DCW^{-1}) compared to overexpression of *crtE* alone. However, overexpression of *gps*, which converts IPP directly to GGPP, together with *idi* increased astaxanthin production even further to 1,418.8 μg g DCW^{-1}. Because *gps* has been cloned from a hyperthermophilic organism, both expression and activity are most likely only suboptimal in *E. coli*. Hence, the same authors subjected *gps* to in vitro evolution and screened for improved variants that further increase carotenoid biosynthesis through the reconstructed isoprenoid pathway. The best *gps* variant containing multiple mutations enhanced lycopene production about 2-fold. Lycopene productivity reached 45 mg g DCW^{-1} when *dxs* was overexpressed together with the mutant *gps* (Wang et al. 2000).

Farmer and Liao (2001) reasoned that balancing the supply of the immediate precursors of the non-mevalonate pathway – pyruvate and glyceraldehyde-3-phosphate (G3P) (the latter being less available in the cell compared to pyruvate as a major metabolic intermediate) (Fig. 1) – would significantly improve carotenoid production. In fact, redirection of the flux from pyruvate to G3P either by overexpression of phosphoenolpyruvate synthase (*pps*) or phosphoenolpyruvate carboxykinase (*pck*) or by inactivation of pyruvate kinase (*pyk*) improved lycopene production in the best case (*pps* overexpression) 5-fold. Placement of *idi* and *pps* expression under dynamic control of an engineered intracellular control loop that senses excess glycolytic flux and in turn up-regulates carotenoid production by inducing *idi* and *pps* expression, led to a significant further increase of lycopene production due to effective utilization of excess pyruvate (Farmer and Liao 2000).

Balancing expression of carotenogenic genes

Balancing the expression of rate-limiting isoprenoid and carotenoid biosynthetic enzymes has been effective in increasing the flux through the carotenoid pathway by relieving growth inhibition caused by overexpression of enzymes (Jones et al. 2000; Kim and Keasling 2001). For example, the utilization of a low-copy instead of a high-copy number plasmid for arabinose-inducible expression of the rate-limiting enzyme DXS enhanced lycopene production 2 to 3-fold (Jones et al. 2000). This demonstrates that overexpression of *dxs* at intracellular concentrations that exceed the availability of its glycolytic substrates pyruvate and G3P represents a significant metabolic burden for the cell. Similarly, cell growth and production of non-native compounds were balanced by induction of biosynthetic gene expression in response to precursor availability using an engineered regulatory dynamic control circuit as described above (Farmer and Liao 2000).

Modulation of expression levels of enzymes can also be used as a strategy to achieve accumulation of different ratios of carotenoid intermediates by controlling the metabolic flux through the carotenoid pathway. Engineering the mRNA stability of two genes encoding phytoene desaturase and lycopene cyclase, varied the production levels of β-carotene relative to lycopene 300-fold and intermediates such as phytoene accumulated that are not observed in *E. coli* cells expressing the recombinant *Erwinia* carotenoid operon (Smolke et al. 2001).

The importance of intracellular biosynthetic enzyme activities for intermediate accumulation and terminal product formation is also reflected by the different synthetic routes observed during in vitro and in vivo astaxanthin formation leading to the accumulation of different intermediates (Kajiwara et al. 1995; Misawa et al. 1995b).

Selection of heterologous host systems

Despite the recent accomplishments in metabolic engineering of *E. coli* cells for carotenoid production, production levels are not yet competitive with carotenoid levels presently produced by fermentation, synthesis or isolation (Johnson and Schroeder 1995). Although one of the major limitations for carotenoid production in *E. coli*, supply of isoprenoid precursors, has been addressed by metabolic engineering, solutions for the second major limitation, inadequate storage capability for lipophilic compounds, have yet to be found.

In contrast to *E. coli*, yeasts exhibit an efficient isoprenoid metabolism and are capable of accumulating large quantities of the triterpenoid ergosterol in their membranes. Furthermore, many yeast strains are considered GRAS organisms, which is desirable for the production of carotenoids for pharmaceutical, nutritional and feed applications. Although metabolic engineering of recombinant yeasts for carotenoid production has not yet been explored widely, the few published examples of using engineered yeasts as heterologous hosts for carotenoid production are promising. For instance, the non-carotenogenic yeasts *S. cerevisiae* (Yamano et al. 1994) and *Candida utilis* (Miura et al. 1998a, b) were successfully engineered to produce lycopene, β,β-carotene, and astaxanthin by diverting the ergosterol pathway with carotenogenic genes derived from *Erwinia*. Overexpression

of HMG-CoA reductase (Fig. 1), which is considered the rate-limiting enzyme in ergosterol biosynthesis, and blockage of ergosterol synthesis by disruption of *erg*9, encoding squalene synthase, yielded a lycopene-overproducing *C. utilis* strain (7.8 mg g DW^{-1}) (Shimada et al. 1998).

Metabolic engineering of photosynthetic bacteria, which already synthesize significant amounts of carotenoids in their intracellular membranes (Harker and Hirschberg 1998), could provide an alternative route for the heterologous production of carotenoids. In fact, some of the earliest examples to reroute carotenoid pathways were carried out by splicing the pathways from *Erwinia herbicola* (*crtB*, *crtI*, *crtY* and *crtZ*) and *Rhodobacter sphaeroides*, resulting in the production of the *Erwinia* carotenoids lycopene, β-carotene and zeaxanthin in *Rhodobacter*, which typically produces the acyclic xanthophylls spheroidene and spheroidenone from neurosporene (Hunter et al. 1994).

Synthesis of spirilloxanthin and ketospirilloxanthin from lycopene in engineered *R. sphaeroides* cells has been achieved by substitution of its three-step desaturase with a four-step desaturase from *Erwinia* (Ausich 1994; Garcia-Asua et al. 2002). More recently, carotenoid biosynthesis in recombinant *Synechocystis* sp. has been manipulated. Overexpression of genes encoding *idi*, β-carotene hydroxylase (*crtR*), phytoene synthase (*crtB*) and phytoene desaturase (*crtP*) in different combinations in a *Synechocystis* sp. strain with a functional or disrupted *crtO*, changed both the ratio and quantity of the synthesized carotenoids (Lagarde et al. 2000). In a different example, overexpression of an algal carotene oxygenase (*crt*O/*bkt*) in a recombinant *Synechocystis* strain, which contains the β-carotene hydroxylase gene for zeaxanthin synthesis, resulted in the production of astaxanthin (Harker and Hirschberg 1997).

Metabolic engineering of carotenoid diversity

The recombinant production of carotenoids in non-carotenogenic microorganisms allows engineering of new pathways for the production of diverse carotenoid structures. Combining carotenoid genes from different sources and altering the activity of carotenoid enzymes by directed evolution have created new carotenoid pathways.

Gene combination

Numerous examples over the last years demonstrate that *crt* genes from different sources can be combined into new functional biosynthetic pathways in a number of heterologous hosts (Table 2). Enzymes from different species can therefore function cooperatively and exhibit sufficient promiscuity regarding the structure of their substrates.

Many carotenoid enzymes, in particular those involved in the later steps of the pathway, are promiscuous regarding their substrates and appear to recognize a particular carotenoid end-group rather than the entire structure. This has been observed in a number of carotenoid gene combinations. For example, lycopene cyclases from *E. uredevora* and *C. annuum* have been shown to cyclize neurosporene and ζ-carotene in addition to their natural substrate lycopene (four desaturations, Fig. 2) when combined with three- and two-step phytoene desaturases (Takaichi et al. 1996). Further extension of the pathway for the synthesis of the cyclic neurosporene derivative with the *crtZ* gene (see Table 1) then produced additional new hydroxy carotenoids in *E. coli*. Various unusual acyclic and cyclic hydroxy carotenoids with enhanced antioxidant properties could be synthesized by combining the phytoene pathway with either three-, four- or five-step desaturase genes and extension with the carotenoid genes *crtC*, *crtD*, *crtY*, *crtZ* and *crtC* (Albrecht et al. 2000). Combination of β- and ε-cyclase genes from *Arabidopsis thaliana* together with carotenoid genes for lycopene and neurosporene synthesis, enabled the synthesis of β- and ε,ψ-carotene and α- and β-zeacarotene in *E. coli*. Co-expression of both cyclases in lycopene-producing *E. coli* cells resulted in the synthesis of α-carotene (Cunningham et al. 1996).

In phototrophic bacteria, the same enzymes (crtC, crtD and crtF) have been shown to synthesize xanthophylls from either lycopene or neurosporene and the order of modifications catalyzed by those enzymes is also not fixed (Albrecht et al. 1997; Komori et al. 1998; Ouchane et al. 1997).

Directed evolution

Combining available biosynthetic genes from different organisms into pathways and altering the catalytic functions of selected enzymes by in vitro evolution (for a review see Schmidt-Dannert 2001) can greatly expand the recombinant production of known and completely new carotenoid structures. We have introduced the pathway for phytoene into *E. coli* and extended this pathway with a library of two shuffled desaturase genes from *Erwinia* strains, which normally catalyze the desaturation of phytoene to lycopene (Schmidt-Dannert et al. 2000). Among the thousands of colored variants screened, several yellow variants were found that introduced fewer desaturations into phytoene and one pink clone was identified that produced the fully conjugated linear carotenoid tetradehydrolycopene. We then extended the evolved tetradehydrolycopene pathway with a library of shuffled cyclase genes, which usually cyclize lycopene to form β-carotene, and obtained a kaleidoscope of different colored variants. Many of the new variants accumulated different ratios and quantities of acyclic and cyclic carotenoid intermediates, but one bright-red variant contained a novel pathway for the production of torulene in *E. coli*. Extension of the evolved torulene pathway in *E. coli* with additional carotenoid biosyn-

Table 2 Combinatorial carotenoid biosynthesis in heterologous hosts (*Escherichia coli* and yeasts) and the resulting major carotenoid accumulated. Subscripts indicate the organisms from which the carotenogenic genes (refer to Table 1 for gene designations) have been cloned: *EU Erwinia uredevora*, *EH Erwinia herbicola*, *SY Synechocystis* sp., *RC Rhodobacter capsulatus*, *RS Rhodobacter sphaeroides*, *CA Capsicum annuum*, *AA Agrobacterium aurantiacum*, *HP Haematococcus pluvialis*, *AN Anabaena*, *AL Alcaligenes* sp., *AT Arabidopsis thaliana*, *XD Xanthophyllomyces dendrorhous*, *SA Staphylococcus aureus*, *CG Corynebacterium glutamicum*

Gene combination[a]	Major carotenoid	Reference
E. coli		
$crtM_{SA} + crtN_{SA}$	4,4′-Diaponeurosporene[a]	Wieland et al. 1994
$crtM_{SA} + crtN_{SA} + crtI_{EU}$	Diapolycopene[a]	Raisig and Sandmann 2001
$crtM_{SA} + crtN_{SA} + crtQ_{AN}$	Diapolycopene[a]	Raisig and Sandmann 2001
$crtE,B_{EU} + crtI_{EU}$	Lycopene	Misawa et al. 1990
$crtE,B_{EU} + crtI_{XD} + crtX_{EU}$	Lycopene	Verdoes et al. 1999
$crtE,B_{EU} + crtI_{EU} + crtY_{EU}$	β-Carotene	Misawa et al. 1990
$crtE,B_{EH} + crtI_{EH} + crtL\text{-}b_{AT}$	β-Carotene	Cunningham et al. 1996
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtC_{RC}$	β-Carotene	Albrecht et al. 1997
$crtE,B_{EU} + crtI_{SY} + crtY_{EU}$	ζ-Carotene	Takaichi et al. 1996
$crtE,B_{EH} + crtI_{SY} + crtL\text{-}b_{AT}$ *or* $crtL\text{-}e_{AT}$	ζ-Carotene	Cunningham et al. 1996
$crtE,B_{EH} + crtI_{EH} + crtL\text{-}e_{AT}$	ε,ψ-Carotene	Cunningham et al. 1996
$crtE,B_{EH} + crtI_{EH} + crtL\text{-}b_{AT} + crtL\text{-}e_{AT}$	ε,ψ-Carotene	Cunningham et al. 1996
$crtE,B_{EH} + crtI_{RC} + crtL\text{-}b_{AT}$	β-Zeacarotene	Cunningham et al. 1996
$crtE,B_{EH} + crtI_{RC} + crtL\text{-}e_{AT}$	Neurosporene	Cunningham et al. 1996
$crtE,B_{EU} + crtI_{RC} + crtY_{EU}$ *or* $crtL\text{-}b_{CA}$	Dihydro-β-carotene	Takaichi et al. 1996
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{EU}$	Zeaxanthin	Misawa et al. 1990
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{AA}$	Zeaxanthin	Misawa et al. 1995b
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{HP}$	Zeaxanthin	Linden 1999
$crtE,B_{EU} + crtI_{XD} + crtY_{EU} + crtX_{EU} + crtZ_{EU}$	Zeaxanthin	Verdoes et al. 1999
$crtE,B_{EU} + crtI_{EU} + crtC_{RS} + crtY_{EU} + crtZ_{AA}$	Zeaxanthin	Albrecht et al. 2000
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{EU} + crtX_{EU}$	Zeaxanthin-β-diglucoside	Misawa et al. 1990
$crtE,B_{EU} + crtI_{RC} + crtY_{EU} + crtZ_{EU}$	Dihydrozeaxanthin	Takaichi et al. 1996
$crtE,B_{EU} + crtI_{RC} + crtY_{EU} + crtZ_{EU}$	7,8-Dihydrozeaxanthin	Albrecht et al. 1997
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtO_{SY}$	Echinenone	Fernandez-Gonzalez et al. 1997
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + bkt_{HP}$	Canthaxanthin	Lotan and Hirschberg 1995
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtW_{AA}$ or $crtW_{EU}$	Canthaxanthin	Misawa et al. 1995a
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtW_{AA}$	Canthaxanthin	Misawa et al. 1995b
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{HP} + bkt_{HP}$	Canthaxanthin	Linden 1999
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{EU} + crtX_{EU} + crtW_{AA}$	Astaxanthin-β-glucoside	Yokoyama et al. 1998
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{AA} + crtX_{EU} + crtW_{AA}$	Astaxanthin	Yokoyama et al. 1998
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{EU} + bkt_{HP}$	Astaxanthin	Kajiwara et al. 1995
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{EU} + crtW_{AA}$	Astaxanthin	Misawa et al. 1995b
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{AA} + crtW_{AA}$	Adonixanthin	Misawa et al. 1995b
$crtE,B_{EU} + Al\text{-}I + crtC_{RS}$	1-HO-3′,4′-Didehydrolycopene	Albrecht et al. 2000
$crtE,B_{EU} + crtI_{EU} + crtC_{RS} + crtD_{RS}$	1,1′-$(HO)_2$-Tetradehydro lycopene	Albrecht et al. 2000
$crtE,B_{EU} + crtI_{EU} + crtC_{RC}$	1,1′-Dihydroxylycopene	Albrecht et al. 1997
$crtE,B_{EU} + crtI_{RC} + crtC_{RC}$	Hydroxyneurosporene	Albrecht et al. 1997; Fernandez-Gonzalez et al. 1997
$crtE,B_{EU} + crtI_{RC} + crtC_{RC} + crtD_{RS}$	Demethylspheroidene	Albrecht et al. 1997
$crtE,B_{EU} + crtI_{EU} + crtEb_{CG}$	Flavuxanthin[b]	Krubasik et al. 2001a
$crtE,B_{EU} + crtI_{EU} + crtEb_{CG} + crtYe_{CG} + crtYf_{CG}$	Decaprenoxanthin[b]	Krubasik et al. 2001a
Yeasts		
$crtE,B_{EU} + crtI_{EU}$	Lycopene[c]	Miura et al. 1998b
$crtE,B_{EU} + crtI_{EU} + crtY_{EU}$	β-Carotene[c]	Miura et al. 1998b
$crtE,B_{EU} + crtI_{EU} + crtY_{EU} + crtZ_{AA} + crtW_{AA}$	Astaxanthin[c]	Miura et al. 1998b
$crtE,B_{EU} + crtI_{EU}$	Lycopene[d]	Miura et al. 1998a
$crtE,B_{EU} + crtI_{EU} + crtY_{EU}$	β-Carotene[d]	Miura et al. 1998a

[a] C30 carotenoids
[b] C50 carotenoids
[c] *Candida utilis* as a host
[d] *Saccharomyces cereviseae* as a host

thetic genes (*crtO*, *crtZ*, *crtX*, etc.) resulted in the production of a variety of new carotenoid structures (e.g., ketotorulene, hydroxytorulene, glycosylated torulene) in *E. coli* (P.C. Lee, R. Momen and C. Schmidt-Dannert, unpublished data). This shows that combinatorial and evolutionary approaches together represent a powerful strategy for the recombinant synthesis of numerous new carotenoid structures.

Recently, the three-step phytoene desaturase crtI from *Rhodobacter* was changed into a four-step desaturase, which efficiently synthesizes lycopene instead of neurosporene, by two generations of random mutagenesis followed by recombination of the best mutants isolated after each round (Wang and Liao 2001). In vitro evolution was also successfully applied to increase the activity of *A. fulgidus* gps as described earlier (Wang et al. 2000).

Conclusions and perspectives

The large pool of available carotenoid biosynthetic genes from different organisms, which can be functionally combined into recombinant pathways, opens numerous possibilities for the synthesis of structurally diverse carotenoids in recombinant microorganisms. In fact, recombinant biosynthesis of carotenoids presently represents the best example for combinatorial biosynthesis, which involves the combination of non-modular single enzymes into new pathways. In vitro evolution of carotenoid enzymes will broaden the diversity of heterologously produced carotenoid structures even further and, in addition, will allow the synthesis of carotenoids not found in nature.

Recent accomplishments in metabolic engineering of the isoprenoid precursor pool and flux through the carotenoid pathway in *E. coli* for increased carotenoid production, have detailed a number of bottlenecks and rate-limiting steps in microbial carotenoid biosynthesis. Carotenoid production in a non-carotenogenic microorganism such as *E. coli* might perturb other, as yet unknown, metabolic pathways that could be identified by gene expression profiling and then become the targets of future metabolic engineering.

Nevertheless, a systematic flux analysis of carotenoid biosynthesis, including the determination of individual enzyme activities and functional expression levels, has not yet been carried out. Only very little is known about the regulation of carotenoid biosynthetic pathways, the interaction and cellular localization of the different enzymes of carotenoid pathways in either natural or recombinant hosts and how physiological and/or environmental factors affect carotenoid biosynthesis. Thus, understanding the biochemical mechanisms governing carotenoid pathway flux is the key to successful future metabolic engineering of microbial carotenoid production. The limited carotenoid storage capability in current heterologous hosts remains an additional major obstacle for achieving commercially relevant carotenoid production levels. Hosts other than *E. coli*, e.g., yeasts, should therefore be considered for future engineering of microbial carotenoid production. Alternatively, engineering of carotenoid sequestering systems into *E. coli* should boost carotenoid production to economical yields.

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