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MINI-REVIEW

Metabolic engineering for the microbial production of carotenoids and related products with a focus on the rare C50 carotenoids

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Abstract Carotenoids, a subfamily of terpenoids, are yellow- to red-colored pigments synthesized by plants, fungi, algae, and bacteria. They are ubiquitous in nature and take over crucial roles in many biological processes as for example photosynthesis, vision, and the quenching of free radicals and singlet oxygen. Due to their color and their potential beneficial effects on human health, carotenoids receive increasing attention. Carotenoids can be classified due to the length of their carbon backbone. Most carotenoids have a C40 backbone, but also C30 and C50 carotenoids are known. All carotenoids are derived from isopentenyl pyrophosphate (IPP) as a common precursor. Pathways leading to IPP as well as metabolic engineering of IPP synthesis and C40 carotenoid production have been reviewed expertly elsewhere. Since C50 carotenoids are synthesized from the C40 carotenoid lycopene, we will summarize common strategies for optimizing lycopene production and we will focus our review on the characteristics, biosynthesis, glycosylation, and overproduction of C50 carotenoids.

Keywords Metabolic engineering of carotenoids · C50 carotenoids · Lycopene elongase · C50 carotenoid cyclase · C50 carotenoid glucosyltransferase

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Introduction

Carotenoids can be differentiated into carotenes that are hydrocarbons like lycopene or β-carotene, and their oxygenated derivatives, the so-called xanthophylls, like, e.g., lutein, zeaxanthin, and astaxanthin (Das et al. 2007). Natural products in general and carotenoids in particular receive increasing attention due to their interesting pigment properties and their potential beneficial effects on human health (Armstrong 1994; Das et al. 2007; Sandmann 2001; Umeno et al. 2005). In 2010, the total commercial value of carotenoids was reported to be $1.2 billion and an estimated annual growth of 2.3 % of the global market (Cutzu et al. 2013). Chemical synthesis of carotenoids from petrochemical precursors has been described in some cases, and e.g., eco-efficient production of enantiopure (3S,3′S) astaxanthin from α-isophorone and vinylbutinol has been commercialized (Jackson et al. 2008). Although numerous microbial carotenoids have been identified, only a few are produced at an industrial scale and the interest in efficient and environmental-friendly production of carotenoids by microbial hosts is rising (Cutzu et al. 2013; Das et al. 2007; Harada and Misawa 2009; Lee and Schmidt-Dannert 2002).

Biosynthesis and occurrence of carotenoids

All carotenoids are derived from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (Rodriguez-Villalon et al. 2008). Two distinct pathways of IPP synthesis exist, the mevalonic acid (MVA) pathway and non-mevalonate pathway or methylerythritol phosphate (MEP) pathway. The MVA pathway, in which acetyl-CoA is the primary educt, is mainly found in eukaryotes (mammals, fungi, in the cytoplasm of plant cells), archaea, and a limited number of bacteria. In most bacteria as well as in plant plastids, IPP is synthesized from pyruvate and glyceraldehyde

3-phosphate (GAP) via the MEP or Sahm-Rohmer pathway (Daum et al. 2009; Kirby and Keasling 2009; Lange et al. 2000; Lee and Schmidt-Dannert 2002; Rohmer 1999). The C5 compounds IPP and DMAPP are the immediate precursors of subsequent chain elongation reactions leading to the C15 carotenoid precursor farnesyl pyrophosphate (FPP) and the C20 carotenoid precursor geranylgeranyl pyrophosphate (GGPP) (Fig. 1). More than 95 % of all known carotenoids are based on a symmetrical C40 backbone, which is formed by the condensation of two molecules of GGPP (Tobias and Arnold 2006). The C25 and C30 apocarotenoids are cleavage products of oxygenated C40 carotenoids (Saelices et al. 2007). C30 carotenoids occur only in a small group of bacteria such as *Staphylococcus*, *Streptococcus*, *Methylobacterium*, and *Heliobacterium* species. They are synthesized by condensation of two molecules of FPP (Tao et al. 2005; Tobias and Arnold 2006).

The rare C50 carotenoids have been mainly isolated from extremely halophilic archaea (*Halobacteria*, *Halococcus*) (Kelly and Jensen 1967; Pfander 1994) and from Gram-positive bacteria of the order *Actinomycetales* (Netzer et al. 2010) and one Gram-negative *Pseudomonas* strain (Miki et al. 1994). The first C50 carotenoid discovered, decaprenoxanthin, was isolated from *Flavobacterium dehydrogenans* (Liaaen-Jensen et al. 1968). Glucosylated C50 carotenoids have been first described in *Arthrobacter* sp. (Arpin et al. 1972). C50 carotenoids are synthesized by the addition of two DMAPP molecules to the C(2) and C(2′) of the respective C40 carotenoid (Fig. 1). To date, the biosynthesis of three C50 carotenoids has been elucidated: the ε-cyclic C50 carotenoid decaprenoxanthin in *Corynebacterium glutamicum* (Krubasik et al. 2001a); the β-cyclic C50 carotenoid C.p. 450, first isolated from *Corynebacterium poinsettiae* (Norgård et al. 1970), in *Dietzia* sp. CQ4 (Tao et al. 2007); and the γ-cyclic C50 carotenoid sarcinaxanthin in *Micrococcus luteus* NCTC2665 (Netzer et al. 2010).

An increasing number of carotenogenic genes are identified, often employing functional expression in heterologous hosts (Lee and Schmidt-Dannert 2002). However, the huge variety of carotenoid biosynthesis enzymes can be assigned to a few classes such as for example carotenoid desaturases, cyclases, or synthases (Umeno et al. 2005). Basically, the carotenoid biosynthetic pathways can be structured in a tree-like hierarchy (Umeno et al. 2005), where the trunk represents the backbone synthesis and the branches represent the various modification steps present in the different species (Armstrong and Hearst 1996). Based on this principle, novel carotenoids were produced by co-expression of biosynthetic genes in combinations not found in nature (Song et al. 2013; Tobias and Arnold 2006), by employing mutated carotenogenic enzymes or by combining both approaches (Tobias and Arnold 2006). In most bacteria, the carotenogenic genes are organized in a gene cluster. *C. glutamicum* possesses a second gene cluster encoding an alternative functional phytoene synthase (Heider et al. 2012) (see "Biosynthesis of C50 carotenoids involves novel elongases, cyclases, and glucosyl transferases" section).

Biological functions, chemical properties, and applications of carotenoids

Due to their health benefits and their possible medicinal, pharmaceutical, and nutraceutical applications, carotenoids come more and more into focus. Commercially available

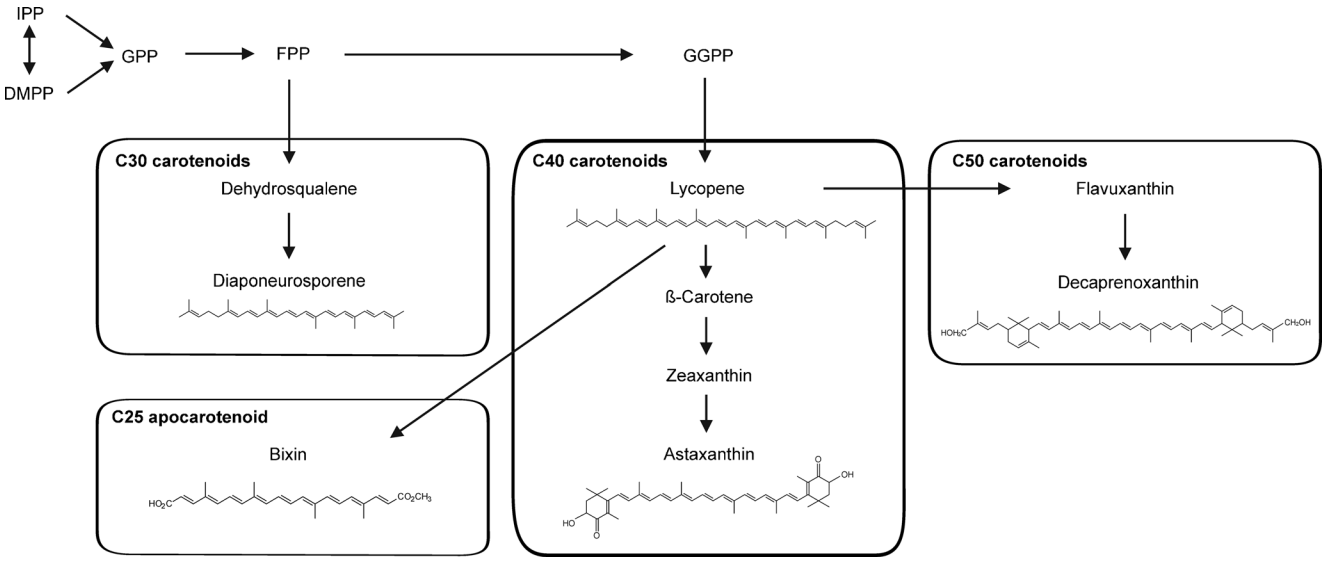

Fig. 1 Schematic diagram of biosynthesis of C30, C40, and C50 carotenoids and C25 apocarotenoids. Selected carotenoids and their structures are given. *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate

carotenoids are mainly used as food and feed colorants with, e.g., up to 130 tons of astaxanthin being used annually in aquaculture and poultry industry (Gassel et al. 2013; Johnson and Schroeder 1996). Furthermore, carotenoids find application for coloration of beverages and in the cosmetic industry (Dembitsky 2005; Downham and Collins 2000).

The high abundance of different carotenoids throughout nature reflects the wide variety of their biological functions. Apart from their obvious role as visually attractive pigments, carotenoids show beneficial or even essential properties for some organisms. The biological functions of carotenoids either involve interactions with light or not (Britton 2008). Absorption of the high-energy part of visible light (400–500 nm) is due to their conjugated polyene backbone. Thus, in photosynthetic organisms, carotenoids contribute to the light harvesting process and additionally serve as photoprotectants by quenching excess light energy and by preventing the formation and reaction of reactive oxygen species (Britton 2008; Johnson and Schroeder 1996). Zeaxanthin, for example, plays an important role in nonphotochemical quenching in algae and higher plants (Pinnola et al. 2013), as a blue-light receptor with relevance to phototropism in corn coleoptiles (Quinones and Zeiger 1994) and phototaxis in guard cells of *Arabidopsis* (Zeiger and Zhu 1998).

Independent of light, the conjugated double bond system of carotenoids protects against oxidative damage elicited by oxidizing agents and free radicals. Quenching of singlet oxygen species is promoted by an increasing number of conjugated C=C or C=O double bonds, but also functional groups like carbonyl, hydroxyl, exomethylene, or dimethylallyl contribute to the antioxidative properties of carotenoids (Bhosale and Bernstein 2005; Jackson et al. 2008; Miller et al. 1996; Naguib 2000; Osawa et al. 2010). Astaxanthin is one of the most efficient scavengers of reactive oxygen species, whereas β-carotene is the more potent reactive nitrogen species scavenger (Rodrigues et al. 2012). Benefits to human and animal health mainly derive from their activity as antioxidants. In addition, carotenoids are thought to have beneficial effects on the human immune system and to protect against degenerative diseases and cancer (Cooper et al. 1999; Krinsky and Johnson 2005; Ye and Bhatia 2012).

Animals are not able to synthesize carotenoids and require uptake of carotenoids through their diet. In animals, carotenoids are precursors for different hormones (Vershinin 1999), vitamin A, retinal, and retinoic acid, which are important substances for nutrition, vision, and cellular differentiation, respectively (Olson 1993).

Due to the lipophilic character and rigid rod-shaped structure of carotenoids, they are often found in or spanning across membranes which causes membrane stabilization through decreasing water permeability and increased firmness (Britton 2008; Johnson and Schroeder 1996; Vershinin 1999). The reduced membrane fluidity might support resistance to toxic substances, osmotic stresses as well as heat and radiation (Abbes et al. 2013; Johnson and Schroeder 1996). Polar end groups, especially hydroxy groups, as well as the dimension of specific carotenoids determine their orientation in the membrane and their influence on the membrane properties. Carotenes like, e.g., lycopene are lipophilic and accumulate within the hydrophobic core of the membrane, whereas carotenoids with polar residues associate with the polar head groups of the membrane bilayer. Glycosylation of the carotenoid end groups even enhances this association (Britton 2008). Carotenoids with two polar substituents usually span across the membrane likely contributing to rigidity (Britton 2008; Rottem and Markowitz 1979). The length of carotenoids found in different bacteria usually matches the thickness of the membrane, which is reflected by the abundance of C40 carotenoids. Accordingly, the acyclic C50 carotenoid bacterioruberin is predominantly found in the thicker diphytanyl lipid membranes of halophilic archaea that are required for survival in extreme hypersaline and low-temperature environments (Abbes et al. 2013). Sterols are membrane optimizers in mammals and have most likely evolved from carotenoids by changes of only a few enzymatic steps in the biosynthesis pathway (Rohmer et al. 1979).

C50 carotenoids have longer conjugated double bounds than most carotenoids and at least one hydroxyl group. These features contribute to their superior antioxidative properties (Netzer et al. 2010) as sarcinaxanthin and its glucosides showed higher singlet oxygen quenching activities than β-carotene (Osawa et al. 2010). The γ-cyclic C50 carotenoid sarcinaxanthin was described to be an effective UV and visible light filter [US 2013/0078203 A1]. Their golden yellow color, solubility, and stability in oil emulsions add to the attractiveness for usage in cosmetic products with certain attention to sunscreens and other light-protecting cosmetics. Therefore, the C50 carotenoids have high potential for the application in nutraceuticals and pharmaceuticals, as well as in the production of derived products, as the so-called apocarotenoids or norisoprenoids.

Cleavage of carotenoids and resulting (norisoprenoid) products

In nature, many carotenoid-derived molecules (norisoprenoids) occur (Fig. 1). Most relevant for the human diet is vitamin A, which is a cleavage product of β-carotene. For this reason, β-carotene is also called provitamin A. Other norisoprenoids that are frequently encountered by consumers include mostly compounds that contribute to the flavor of food and beverages and to fragrance products. Well-known examples include safranal, which provides the saffron flavor to sauces and paella dishes; bixin, which is the pigment in annatto, used for

coloring dairy products such as cheese; damascenone which is part of many perfumes; and ionones, which is used for flavoring softdrinks, candies, and tobacco (Winterhalter and Rouseff 2001). Other norisoprenoids include plant regulatory molecules. Well-known examples include plant hormones abscisic acid, which inhibits seed germination, induces the loss of leaves upon environmental stress, and regulates stomatal transpiration (Bari and Jones 2009); and strigolactones, which regulate branching of plants and interactions with arbuscular mycorrhiza (Cheng et al. 2013). Also, molecules that signal oxidative stress, such as cyclocitral, derive from carotenoids (Havaux 2013).

Most norisoprenoids are oxidation products of carotenoids from plant materials. They are known to occur mostly during maturation or ripening stages, as the result of breakdown of plastidal carotenoid. This is for example the case during maturation of seeds as in the case of Bixa seeds that produce the annatto color substance bixin (Bouvier et al. 2003). Many fruits are known to derive an important part of their flavor during ripening from norisoprenoids, e.g., raspberry and white-fleshed peaches (Beekwilder et al. 2008; Brandi et al. 2011). For perfumery, it has been long known that the mature flowers from rose, *Osmanthus fragrans*, and *Boronia metastigma* produce abundant amounts of volatile norisioprenoids (Winterhalter and Rouseff 2001), while also fermentation of tobacco leaves produces high amounts of fragrant carotenoid breakdown products (Winterhalter and Rouseff 2001).

In nature, formation of norisoprenoids from carotenoids may occur through photooxidation, or through enzymatic dioxygenation. Photooxidation is at the basis of the formation of cyclocitral during photooxidative stress (Havaux 2013), and the formation of a plethora of norisoprenoids during tobacco fermentation is likely also the result of oxidative breakdown (Winterhalter and Rouseff 2001). When the formation of norisoprenoids is part of programmed breakdown of carotenoids, often a carotenoid cleavage dioxygenase (CCD) is involved. For instance, during fruit ripening of raspberry, the formation of norisoprenoids α- and β-ionone accompanies the strong reductions in carotenoid content, and the upregulation of the expression of a carotenoid cleaves dioxygenase RiCCD1. This RiCCD1 is capable of converting carotenoid into ionones in *Escherichia coli*, by performing symmetric dioxygenation reactions at the 9–10 position (Beekwilder et al. 2008). Likewise, other CCDs have been identified in both plants and mammals which cleave at other positions in the carotenoid and play an essential role in formation of vitamin A, ABA (Schwartz et al. 1997), strigolactones, bixin (Bouvier et al. 2003), and so forth. These CCDs have structural features such as hydrophobic patches, which permit them to access carotenoids in a hydrophobic environment such as membranes, though the enzymes themselves are usually water soluble (Sui et al. 2013).

Clearly, there are industrially relevant compounds among the norisoprenoids, which would be suited for a microbial production system. Currently, norisoprenes are commercially produced by chemical synthesis, since content of plant materials is too low. Alternative production processes include the enzymatic oxidation of carotenoids by lipoxygenase. This enzyme is also used for the enzymatic degradation of carotenoids during the bleaching of whey that has been recovered from cheese production, which is usually stained yellow for cheese coloring. Lipoxygenase will remove the carotenoid-derived yellow color from this whey (De Roos et al. 2005). Industrial production processes using dioxygenases have not yet been implemented at a large scale.

Biosynthesis of C50 carotenoids involves novel elongases, cyclases, and glucosyl transferases

C50 carotenoid-specific elongases and cyclases

Cyclic and acyclic C50 carotenoids are known, e.g., from actinobacteria and halophilic Archaea, respectively, and their biosynthesis proceeds via the C40 carotenoid lycopene by elongation with two C5 units followed by the hydroxylation of the residual ends (Fig. 2) and, in the case of cyclic C50 carotenoids, subsequent cyclization (Fig. 2). Although only a few enzymes involved in the biosynthesis of acyclic and cyclic C50 carotenoids have been described to date, these enzymes are distinct from other prenyltransferases and terpenoid cyclases, respectively. The enzymes for the formation of a cyclic C50 carotenoid were first described for the two-step synthesis of decaprenoxanthin from lycopene in *C. glutamicum* (Krubasik et al. 2001a). First, the consecutive electrophilic attack of a DMAPP cation at the C1,2 double bond or C1′,2′, respectively, is catalyzed by the lycopene elongase, encoded by *crtEb* (Fig. 2). The hydroxylation at C1 position is suggested to stabilize the formation of the prenylated carbocation. Subsequently, the cyclization of the linear C50 intermediate flavuxanthin forming a C1,6 bond is catalyzed by the heterodimeric C50 ε-cyclase, which consists of two polypeptides encoded by $crtY_e$ and $crtY_f$. Similarly, sarcinaxanthin biosynthesis proceeds via flavuxanthin followed by γ-ring formation at the C1,6 position, which is catalyzed by CrtE2 and $CrtY_{g/h}$. Both heterodimeric C50 cyclases were shown to be restricted to C50 backbones as substrates since they were not able to use lycopene as substrate (Krubasik et al. 2001a; Netzer et al. 2010). In *Dietzia* sp., elongation of lycopene results in a structurally slightly different linear C50 intermediate, C.p. 496, which subsequently is converted to the β-cyclic C50 carotenoid C.p. 450. Interestingly, while *lbtA* encodes one subunit of the C50 β-cyclase, the other subunit is fused to lycopene elongase encoded by *lbtBC* in this bacterium (Tao et al. 2007).

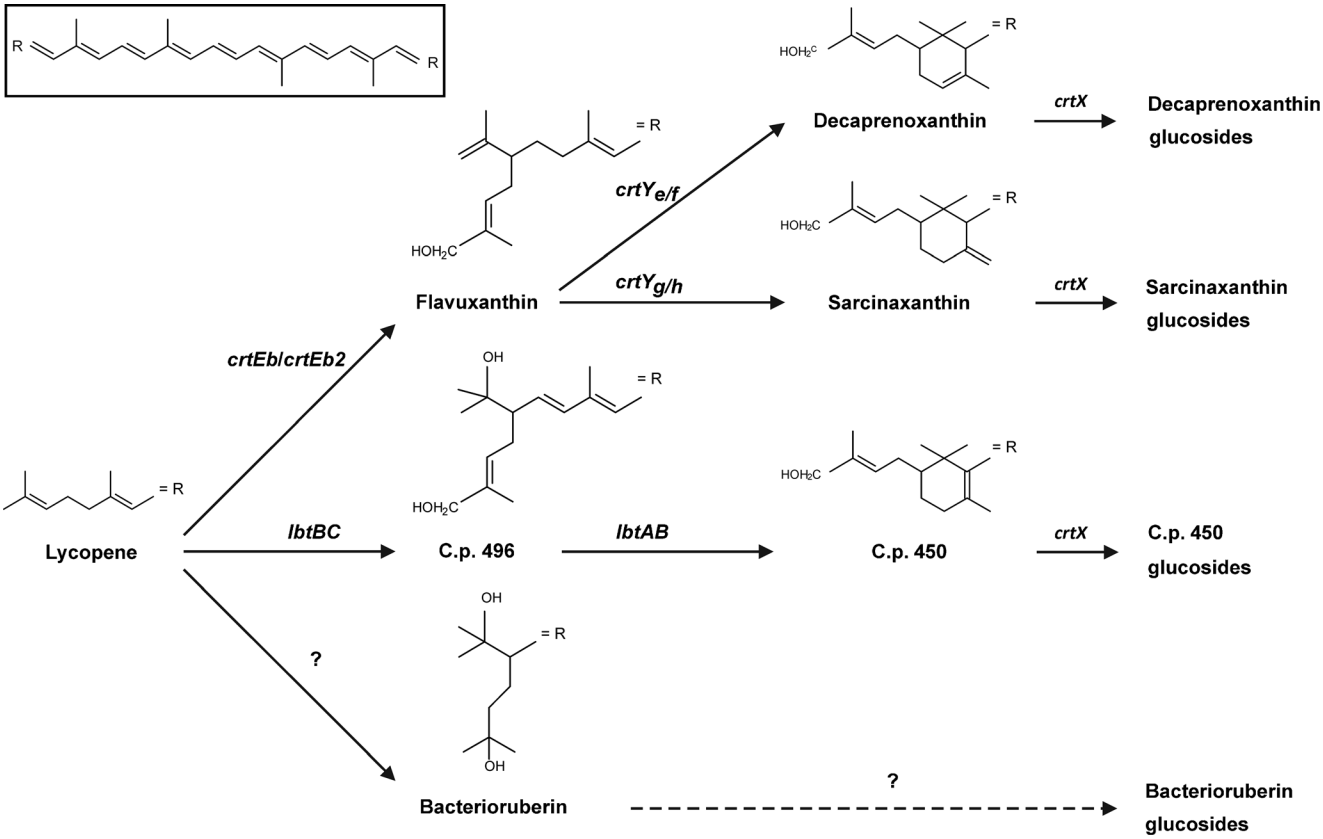

Fig. 2 Biosynthesis of linear and cyclic C50 carotenoids derived from lycopene. The linear carotenoid backbone structure with terminal residues labeled R is shown in a *box* and residues for lycopene, flavuxanthin, decaprenoxanthin, sarcinaxanthin, C.p. 496, C.p. 450, and bacteriorubin are depicted. Gene names for the carotenoid elongase (*crtEb*, *crtEb2*, and *lbtBC*), cyclase (*crtYg/h*, *crtYe/f*, and *lbtAb*), and glucosyltransferase (*crtX*) enzymes are given. *C.p. 496* 2,2′-bis-(3-methylbut-2-enyl)-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene-1,1-diol, *C.p. 450* 2,2′-bis-(4-hydroxy-3-methybut-2-enyl)-β,β-carotene

Lycopene elongases are distinct from other prenyltransferases as is evident from their phylogenetic analysis (Fig. 3). Three clusters can be found: the defined cluster of characterized and putative lycopene elongases (cluster 1 in Fig. 3); the GPP, FPP, and GGPP synthases (cluster 2 in Fig. 3); and the hepta- and octaprenyltransferases of ubiquinone and menaquinone biosynthesis (cluster 3 in Fig. 3). Cluster 1 with the lycopene elongases can be further subdivided into a subcluster representing the respective enzymes from actinobacteria such as CrtEb from *C. glutamicum* (subcluster 1A in Fig. 3) and the putative lycopene elongases from haloarchaea, e.g., from *Natromonas pharaonis* (subcluster 1B in Fig. 3). Thus, lycopene elongases active in the biosynthesis of cyclic C50 carotenoids can be distinguished from lycopene elongases active in the biosynthesis of acyclic C50 carotenoids.

The C50 carotenoid cyclases are heterodimers and their subunits can be distinguished from the C40 β-cyclases and ε-cyclases involved in carotene biosynthesis, e.g., in orange fruits (*Citrus sinensis*) (Fig. 4). The subunits of the heterodimeric C50 carotenoid cyclases fall into two different subclusters, e.g., with $CrtY_e$ from the *C. glutamicum* ε-cyclase and $CrtY_h$ from the *M. luteus* γ-cyclase in subcluster 1A (Fig. 4) and their respective partners $CrtY_f$ and $CrtY_g$ in subcluster 1B (Fig. 4). Based on the observed relationship between the subunits of the C50 cyclases, it appears possible that subunits of different species, such as *C. glutamicum* $CrtY_e$ and *M. luteus* $CrtY_g$, may form an active cyclase; however, this has not yet been tested experimentally.

Glycosylation of C50 carotenoids

Hydroxylated carotenoids can be O-glycosylated, O-acetylated, or esterified differently (Dembitsky 2005; Maresca and Bryant 2006; Takaichi et al. 2001). Despite their widespread occurrence, only a limited number of glycosylated carotenoids have been described (Dembitsky 2005). The first glycosylated carotenoid that had been reported, crocin, was isolated from saffron (Aschoff 1818). The transfer of an activated sugar molecule to the carotenoid is catalyzed by specialized glycosyltransferases, which so far are only described for a few organisms.

C. glutamicum synthesizes the diglucosylated C50 carotenoid decaprenoxanthin (Krubasik et al. 2001b). Recently, the

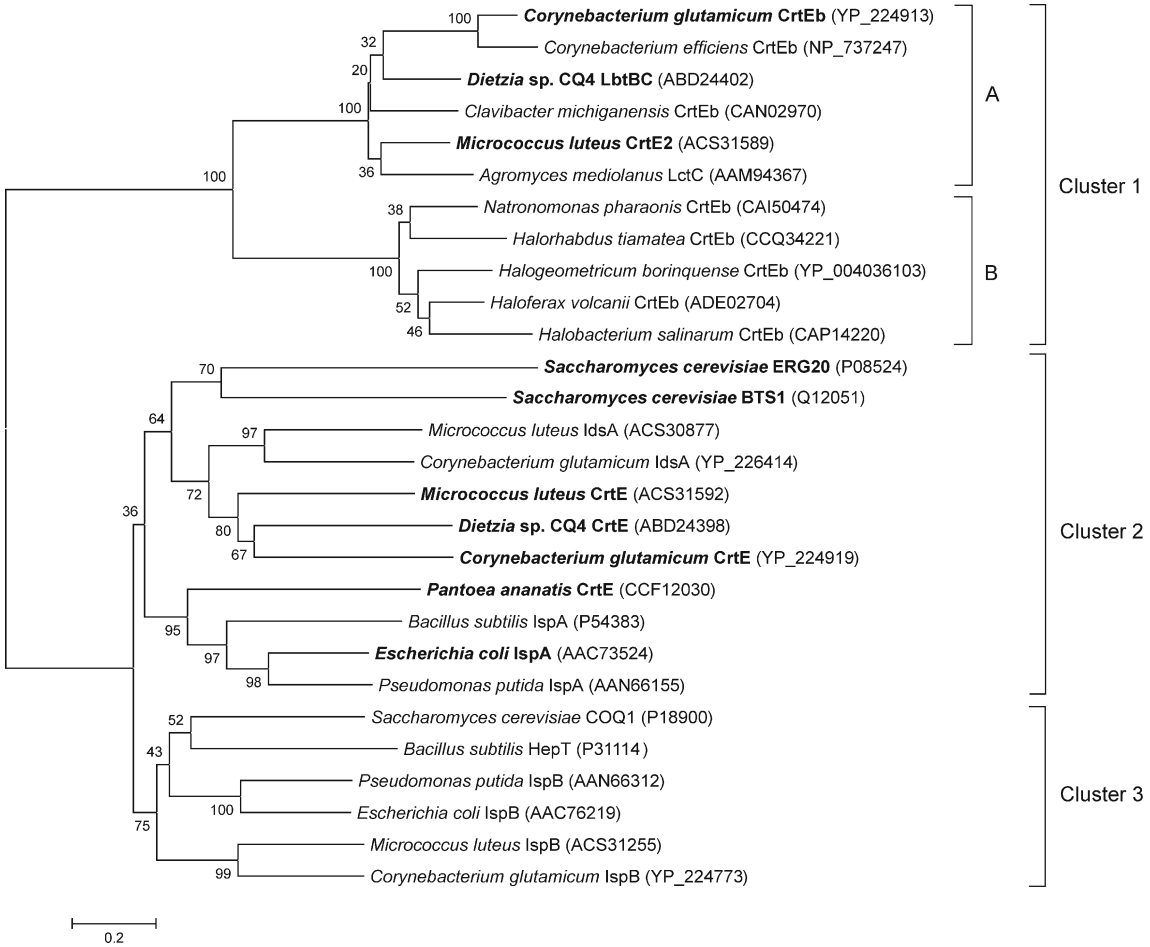

Fig. 3 Phylogenetic relation of prenyltransferases. The sequence alignment and phylogenetic tree analysis was performed with MEGA version 5.2 using Clustal W and the neighbor-joining method. Cluster 1 highlights C50-specific lycopene elongases of actinobacteria (subcluster A) and halophilic archaea (subcluster B). Cluster 2 highlights GPP, FPP, and GGPP synthases (subclusters C and D). Cluster 3 highlights hepta- and octaprenyltransferases of the menachinone/ubichinone pathway

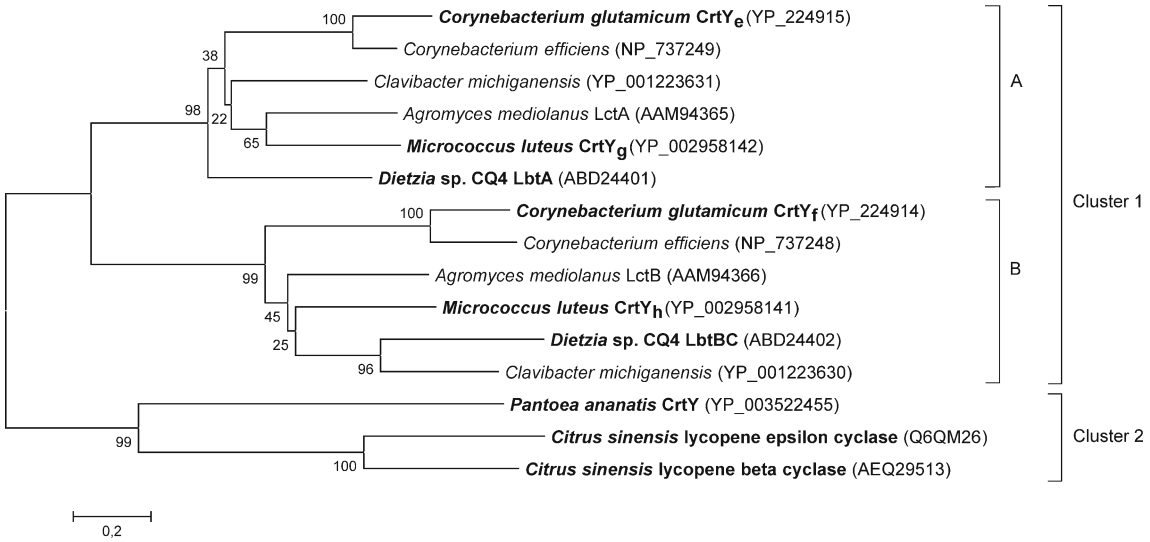

Fig. 4 Phylogenetic relation of carotenoid cyclases. The sequence alignment and phylogenetic tree analysis was perfomed with MEGA version 5.2 using Clustal W and the neighbor-joining method. Cluster 1 with subclusters A and B highlights C50-specific carotenoid cyclases and cluster 2 exhibits an outgroup of lycopene cylases leading to carotene derivatives

gene encoding a glucosyl transferase was identified and characterized to also glucosylate two further C50 carotenoids, sarcinaxanthin and C.p. 450 (Heider et al. 2013). The *crtX* gene of *C. glutamicum* showed overall identities on amino acid level of 33 % to the *M. luteus* gene (90/274), which was the first gene described encoding a functional C50 carotenoid glucosyl transferase (Netzer et al. 2010). For *Dietzia* sp. CQ4, a putative *crtX* gene has been proposed to be involved in the glucosylation of the C50 β-ring carotenoid C.p. 450. While the *crtX* genes of *M. luteus* and *Dietzia* are located in the immediate vicinity of the other carotenoid genes (Netzer et al. 2010; Tao et al. 2007), the carotenoid cluster of *C. glutamicum* does not contain the *crtX* homologue, but it is separated by about 6 kb (Heider et al. 2013).

The C50 carotenoid glucosyl transferases differ from the well-characterized C40 zeaxanthin glucosyl transferases as the respective proteins are much shorter and share less than 14–19 % sequence identity. Glycosyl transferases can be clustered in two distinct structural folds, GTA and GTB. While CrtX from *C. glutamicum*, *Dietzia*, and *M. luteus* belong to the GTA domain structure, CrtX from *Pantoea ananatis* belongs to the GTB domain structure of the family 1 type (Coutinho et al. 2003). A phylogenetic analysis of the so far characterized carotenoid glycosyltransferases (Fig. 5) revealed three distinct clusters. Cluster 1 comprises CrtX from *C. glutamicum*, *Dietzia*, and *M. luteus*, GT2-family glucosyltransferases specific for C50 carotenoids. Cluster 2 comprises GT2-family glycosyltransferases active with various acceptor and donor molecules. *Chlorobium tepidum* CruC, for example, converts the monocyclic C40 carotenoid chlorobactene to its monoglucoside (Maresca and Bryant 2006), while CruG from *Synechoccus* glycosylates only the 2′-hydroxylgroup at the linear end of the monoyclic myxol, resulting in the monoglucoside myxol-2′ fucoside (Graham and Bryant 2009). The third cluster in Fig. 5 comprises the GT1-family zeaxanthin glycosylases from *Enterobacteriaceae bacterium* (Sedkova et al. 2005), *Pantoea agglomerans* (formerly *Erwinia herbicola*; To et al. 1994) and *P. ananatis* (Misawa et al. 1990). Recently, it could be shown that CrtX from *P. ananatis* is characterized by a broader substrate range accepting C50 carotenoids and linear as well as cyclic hydroxyl functions as substrate (Heider et al. 2013).

Metabolic engineering and synthetic pathways for heterologous production of natural and novel C30/C40 carotenoids

With an increasing interest for naturally produced carotenoids, investigations on large-scale production in microbial hosts have been made and are still ongoing to improve processes. The overproduction of carotenoids was studied in natural carotenoid producers as well as noncarotenogenic hosts with a biotechnological relevance like *E. coli* and *Saccharomyces cerevisiae*. The engineering of those organisms toward lucrative carotenoid production would require the optimization of the isoprenoid precursor supply, the balanced expression of the suitable target genes, and the provision of storage capacities for the lipophilic products as well as access to cheap

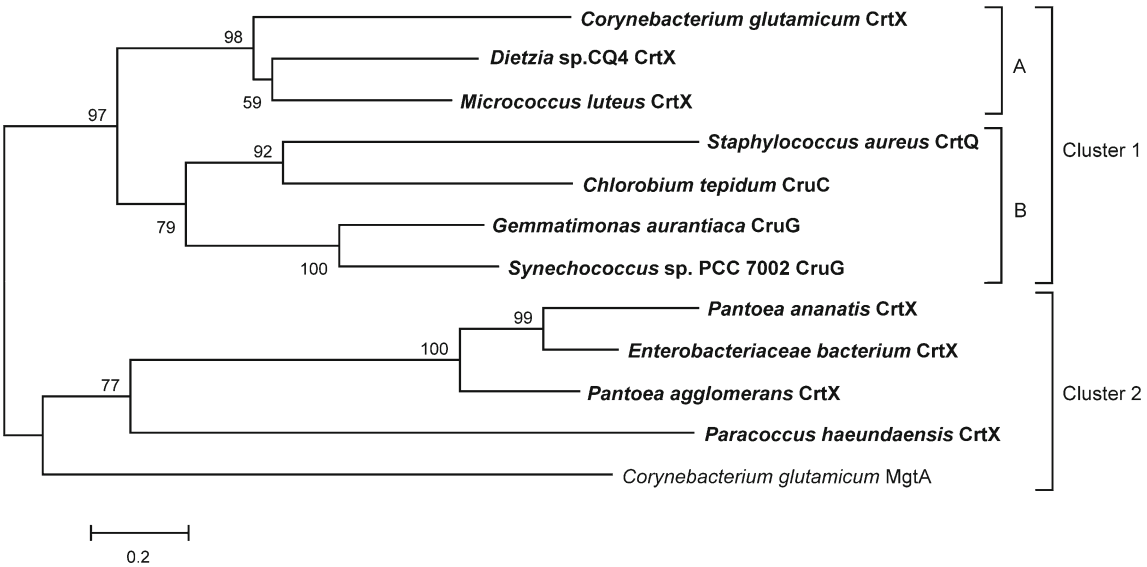

Fig. 5 Phylogenetic relation of carotenoid glycosyltransferases. Modified from Heider et al. (2013). The sequence alignment was carried out using Clustal Omega, the phylogenetic tree analysis was performed with MEGA version 5, and the phylogenetic tree was constructed using the neighbor-joining method. The functionally unrelated GDP-mannose-dependent alpha mannosyltransferase MgtA from *C. glutamicum* (Tatituri et al. 2007) was used as outgroup. Cluster 1 with subclusters A and B highlights GT-A glycosyltransferases and cluster 2 highlights GT-B glycosyltransferases

carbon sources. The common means and achievements in engineering of production of the universal carotenoid precursor IPP and of the C50 carotenoid precursor lycopene and its derivatives will be the focus of this section.

All carotenoids derive from the universal precursor IPP and its isomer DMAPP. The efficient supply of these precursors represents one of the major approaches of metabolic engineering for microbial carotenoid production. There are two distinct routes leading to IPP, the mevalonate and the MEP pathway, starting from acetyl-CoA and pyruvate and GA3P, respectively, intermediates of the central carbon metabolism. Metabolic engineering of the MEP pathway for efficient supply of IPP and DMAPP was reviewed previously (Lee and Schmidt-Dannert 2002; Das et al. 2007; Harada and Misawa 2009). Alternatively, the introduction of a heterologous mevalonate pathway, bypassing the probably strictly regulated MEP pathway, also yielded an increase in the precursor supply of the recombinant hosts. Overexpression of certain isoprenoid biosynthetic genes in combination with modifications of the central carbon metabolism yielded an additional increase of carotenoid production.

The Gram-negative bacterium *E. coli* is a suitable host for heterologous production of a wide range of both natural and novel C30 and C40 (and C50) carotenoids (Wang et al. 2007). It has well-developed genetic tools enabling advanced metabolic engineering, is relatively solvent tolerant, and is amenable for high-cell-density cultivations (HCDC) needed for industrial upscaling in fermenters. One bottleneck though is the limited available space for their accumulation in the *E. coli* cell membrane leading to restricted carotenoid yields (Das et al. 2007; Harada and Misawa 2009). Much metabolic engineering work in *E. coli* has focused on optimizing the MEP and MVA pathways leading to efficient supply of the precursor molecules IPP and DMAPP, as well as the further condensation of these molecules into the key C40 carotenoid lycopene (see Chen et al. (2013) and references therein). Lycopene production levels up to 33 mg/g dry weight (DW), and production yields up to 220 mg/l under HCDC, have been reported (Alper et al. 2006). Thus, metabolically engineered platform strains have been developed, in particular for C40 and C50 carotenoids, and it has also been demonstrated that the choice of the *E. coli* strain used as genetic background for such engineering approaches can have high impact on the production yields of various carotenoids (Chae et al. 2010). Natural C30 carotenoids that have been heterologously produced in *E. coli* include diaponeurosporene (Fig. 1), diapolycopene, and diapotorulene (Chae et al. 2010) and their derivatives (Tao et al. 2005). A high number of different natural C40 carotenoids have been produced heterologously in *E. coli* (for review, see Harada and Misawa (2009)). Of particular interest here is the construction of hybrid biosynthetic pathways by recruiting carotenogenic genes from various origins leading to the biosynthesis of novel C40 carotenoids. For example, the heterologous expression of various *crtEBI* combinations using genes from different bacterial origins was shown to have an impact on the lycopene biosynthetic pathway in *E. coli*, resulting in novel lycopene derivatives being produced (Song et al. 2013). An analogous study (Kim et al. 2010) showed that a novel lycopene derivative, 3,4-didehydrolycopene, could be synthesized in *E. coli*, and this molecule could be further converted into new novel carotenoids by using heterologous enzymes. High-yield production was not reported so far, but this approach creates the perspective to produce valuable carotenoid structures not found in nature. A common observation is that the specificity of carotenogenic enzymes may appear different in heterologous hosts and combined with other heterologous enzymes, thus, opening for huge possibilities of generating novel carotenoids in *E. coli*.

In the eukaryotic model organism *S. cerevisiae*, many strains optimized for producing terpenoids, e.g., C40 carotenoids, have been engineered. Most successfully, this has been done by introducing three genes from the carotenoid-producing fungus *Xanthophyllomyces dendrorhous*, which lead to the formation of β-carotene (Verwaal et al. 2007). *Xanthophyllomyces* itself produces asthaxanthin, which is a valuable carotenoid for aquaculture. In yeast, carotenoids can be synthesized as an ectopic branch from the pathway toward sterols, the mevalonate pathway. Introduction of CrtE, CrtYB, and CrtI genes from *Xanthophyllomyces*, additional copies of CrtI, and a deregulated enzyme of the mevalonate pathway, carotenoid production in *S. cerevisiae* reached up to 5.9 mg/g DW (Verwaal et al. 2007). Stability of this level of production has been difficult to maintain, due to genetic instability of the strain (Verwaal et al. 2010). Characterization of the carotenoid-producing strain indicated that membrane stress was a major cause of selection pressure for low-producing mutants. Strain stability has later been addressed by other groups and could be enhanced by further changes in the mevalonate pathway (Lange and Steinbüchel 2011). By overexpression of *crtYB*, *crtS*, *crtR*, and *crtI* from *Xanthophyllomyces* and additional copies of endogenous GGPP synthase (BTS1), a baker's yeast producing asthaxanthin has been described, but to a much lower concentration compared to β-carotene (29 μg/g DW; Ukibe et al. 2009). Bacterial genes from *Agrobacterium aurantiacum* have been employed to produce carotenoids such as β-carotene and astaxanthin in the yeasts *Pichia pastoris* (3.7 μg/g DW; Araya-Garay et al. 2012) and *Candida utilis* (1.1 mg/g DW; Miura et al. 1998) and the filamentous fungus *Mucor circinelloides* (250 μg/g DW; Papp et al. 2006). Most terpene biosynthetic pathways have been engineered as a branch from the mevalonate pathway, which normally supplies the ergosterol pathway, and other pathways that start from FPP. The re-routing of this pathway toward novel terpenoids has been achieved very efficiently by several research

groups, mainly with the aim to produce C15 sesquiterpenes. A recent study (Scalcinati et al. 2012) shows interesting approaches to increase the flux toward sesquiterpenes. This study directed engineering to four points in the pathway: The FPP branch point was optimized to maximize the availability of FPP by glucose-sensing regulation of squalene synthase and by knocking out farnesol dephosphorylases. The mevalonate pathway was deregulated by overexpression of a truncated version of the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) enzyme and by overexpression of the farnesyl diphosphate synthase. The availability of NADPH and NADH was balanced by manipulating the expression of different isoforms of glutamate dehydrogenase. A mutant transcription factor that normally regulates sterol biosynthesis was overexpressed. Clear effects of all these approaches were demonstrated. In another study, Westfall et al. (2012) showed engineering of biosynthesis of the sesquiterpene amorphadiene to the level of 40 g sesquiterpene per liter culture. This was achieved by using a glucose-tuned version of the galactose promoter system and expression of the complete mevalonate pathway using this promoter system, three copies of the truncated HMGR, and optimization of the fermentation conditions, using ethanol as a feedstock.

The Gram-positive *Bacillus subtilis*, a well-established model and production organism in particular for proteins (Papagianni 2012), is a promising candidate for the production of pharmaceutical isoprenoids (Zhou et al. 2013), due to its GRAS status and its rapid growth rate. *B. subtilis* has been found to excrete isoprene, and the respective MEP genes for the biosynthesis of the immediate precursor of isoprene, IPP, have been identified (Wagner et al. 2000) and analyzed with respect to their importance for isoprene production (Julsing et al. 2007). All genes of the MEP pathway, except the isoprene isomerase, had been shown to be essential. Furthermore, the importance of the 1-deoxy-D-xylulose-5-phosphate synthase (DXS) catalyzing of the primary step of the MEP pathway was demonstrated: Overexpression of the encoding *dxs* gene resulted in an increased isoprene production of 40 % (Xue and Ahring 2011). The overexpression of the following gene in this pathway, *dxr*, had no effect on production, which is in accordance to the observations made for *E. coli*. The highest reported isoprenoid titer achieved by fine-tuned overexpression of the MEP genes *dxs* and *idi* in combination with the FPP cyclase gene *ads* was about 20 mg/l amorphadiene (Zhou et al. 2013). Cytotoxicity of isoprenoid precursors was shown for *E. coli* (Martin et al. 2003) and *B. subtilis* (Sivy et al. 2011). Since isoprenyl compounds are needed for cell growth (Hess et al. 2013; Julsing et al. 2007), terpenoid biosynthesis and precursor supply need to be fine-tuned for effective metabolic engineering (Zhao et al. 2013). Production of carotenoids by *B. subtilis* has not yet been a major focus; however, production of the C30 carotenoids 4,4′-diapolycopene and 4,4′-diaponeurosporene was accomplished by heterologous expression of the *crtMN* genes from *Staphylococcus aureus* (Yoshida et al. 2009). The recombinant strain was more resistant to oxidative stress due to addition of H_2O_2. Only one report dealt with the transformation of *B. subtilis* with C40 carotenoid genes originating from *P. ananatis*, but carotenoid production could not be achieved (Nishizaki et al. 2007).

The Gram-positive *C. glutamicum* represents another advantageous production host for terpenoids in general or carotenoids in particular, since it is a GRAS organism like *B. subtilis* and has been safely used for more than 50 years at large-scale production in food and feed biotechnology. Furthermore, this bacterium is accessible for metabolic engineering since a well-established tool box is available. *C. glutamicum* is able to utilize a multitude of different carbon sources for growth and production and has been additionally engineered for the consumption of alternative feed stocks like glycerol from the biodiesel process (Meiswinkel et al. 2013), pentoses (Gopinath et al. 2011), amino sugars (Uhde et al. 2013), starch (Seibold et al. 2006), and β-glucans (Tsuchidate et al. 2011). Besides these general considerations for a suitable production host, *C. glutamicum* is a natural producer of the C50 carotenoid decaprenoxanthin and its glucosides. The biosynthetic pathway of decaprenoxanthin had been elucidated in this organism (Krubasik et al. 2001b) and genes of the MEP pathway are annotated in the genome. Engineering for increased precursor supply has not been reported so far. The heterologous expression of *C. glutamicum* carotenogenic genes has been conducted in several studies (Krubasik et al. 2001a; Netzer et al. 2010; Song et al. 2013; Tao et al. 2007). Recently, *C. glutamicum* has been engineered for elevated production of a variety of different carotenoids (Heider et al. 2013). In this approach, the strain engineering focused on the terminal carotenoid pathway as it was shown that these modifications were sufficient to achieve lycopene yields in the milligram per gram DW range (Heider et al. 2012). Based on a lycopene-accumulating platform strain, overproduction of native decaprenoxanthin as well as of foreign C40 and C50 carotenoids up to 3–4 mg/g DW was possible. The genes for the synthesis of decaprenoxanthin from lycopene were deleted in a *C. glutamicum* platform strain (LYC) that accumulated lycopene. Flux toward lycopene was elevated by plasmid-borne overexpression of an artificial operon comprising the native genes *crtE*, *crtB*, and *crtI*. Heterologous expression of the lycopene β-cyclase encoding gene *crtY* from *P. ananatis* yielded 4.0±0.5 mg/g DW of β-carotene (Heider et al. 2013). Upon additional expression of *crtZ*, zeaxanthin accumulated to about 1 mg/g DW (Heider et al. 2013).

Pseudomonas putida is solvent tolerant (Meijnen et al. 2011) and can be used to produce toxic hydrophobic compounds like for example phenol, hydroxybenzoate, or hydroxy-styrene to high quantities without being harmful to the cell (de Bont 1998). Its solvent tolerance allows the in situ

extraction of the either very hydrophobic or volatile products by adding a second organic phase to prevent the accumulation of the product to concentrations that are inhibitory to microorganisms and/or to trap the volatiles (Meijnen et al. 2011). However, when engineering the *P. putida* WT strain KT2440 for the production of zeaxanthin, lycopene toxicity was observed and a lycopene-tolerant *P. putida* strain had to be selected after transposon mutagenesis. The mutant was transformed with a plasmid encoding *crtE*, *crtI*, *crtB*, *crtY*, and *crtZ* from *P. ananatis* in an artificial operon and a second plasmid for rhamnose inducible expression of the *E. coli* isoprenoid synthesis genes *dxs*, *idi*, and *ispA* (geranyl transferase) and accumulated 7 mg/g DW zeaxanthin with a volumetric yield of about 50 mg/l (Beuttler et al. 2011). Of various hydrophobic compounds tested for extraction during fermentation, oleic acid and lecithin were the best yielding 239 mg/l zeaxanthin (Beuttler et al. 2011). With respect to C50 carotenoids, *Pseudomonas* sp. KK10206C is the only Gram-negative bacterium described to produce a C50 carotenoid, the bicyclic C50 carotenoid okadaxanthin. This *Pseudomonas* sp. is associated with a marine sponge. Okadaxanthin is similar to decaprenoxanthin, but unlike decaprenoxanthin biosynthesis, its synthesis from lycopene is proposed to involve a head-to-head condensation. The quenching abilities of okadaxanthin for singlet oxygen are more effective than that of α-tocopherol. The pathway for the production of okadaxanthin and the corresponding enzymes or genes have not been identified in this species so far (Miki et al. 1994).

Microalgae are relevant for carotenoid production including C50 carotenoids since some oleaginous species may accumulate carotenoids to comparably high titers. Mircoalgae have been used to produce the ketocarotenoid asthaxanthin which has a total market of more than 200 Mio $ per year (Li et al. 2011) due to its importance in the pharmaceutical and food and especially in the aquaculture industries (Rodriguez-Saiz et al. 2010). Astaxanthin is the predominant carotenoid in salmon trout and various crustacean species in concentrations of 4 mg/kg flesh responsible for its color and, therefore, is added to salmon aquaculture feed (Margalith 1999). Since the biosynthesis of astaxanthin is restricted to microorganisms, only the green freshwater microalga *Haematococcus pluvialis* and the red yeast *Xanthophyllomyces dendrohous* are hitherto considered as production hosts (Bhosale and Bernstein 2005; Rodriguez-Saiz et al. 2010). *H. pluvialis* can accumulate carotenoids up to 3 % of its dry weight which is a considerably higher amount compared to yeast (Bhosale and Bernstein 2005). Moreover, it produces the (3S,3′S) stereoisomer (Boussiba et al. 2000), which is the predominant form identified in wild salmon, while yeast produces the (3R,3′R) form (Andrewes and Starr 1976). However, growth of algae is in general slow. Only little engineering of *H. pluvialis* has been reported, but mutants resistant to carotenoid biosynthesis inhibitors such as norflurazon or fluoridine (Tjahjono et al. 1994) or to the inhibitor of the isoprenyl biosynthesis compactin, which is a competitive inhibitor of hydroxymethyl-glutaryl-CoA reductase, have been isolated (Chumpolkulwong et al. 1997). Site-directed mutants of phytoene desaturase of *H. pluviali* were used to increase astaxanthin production (Steinbrenner and Sandmann 2006). The biosynthesis of other xanthophylls by microalgae was reviewed in Bhosale and Bernstein (2005) and Margalith (1999).

Metabolic engineering for overproduction and controlled glucosylation of C50 carotenoids

C. glutamicum WT natively produces the C50 carotenoid decaprenoxanthin and its glucosides as predominant carotenoids, and the biosynthetic pathway had been elucidated in this organism by Sandmann and co-workers in 2001 (Krubasik et al. 2001b). The high-yield production of different C50 carotenoids, the native as well as two foreign C50 carotenoids, was achieved by engineering the terminal carotenoid pathway. The synthetic pathways for the production of the various C50 carotenoids were introduced by transformation of *C. glutamicum* LYC (described earlier) with a plasmid encoding the lycopene processing genes. This way the heterologous expression of *crtE2*, *crtYg*, and *crtYh* from *M. luteus* and the codon-optimized genes *lbtA* and *lbtBC* from *Dietzia* sp. CQ4 entailed the production of sarcinaxanthin and C.p. 450 in reasonable amounts of 3.4±0.1 and 2.7±0.2 mg/g DW, respectively. These concentrations of foreign carotenoids are comparable to the one of the native C50 carotenoid decaprenoxanthin accumulating in a decaprenoxanthin overproducing strain (3.9±0.2 mg/g DW), proving that the production is transferable to nonendogenous carotenoids (Heider et al. 2013). To our knowledge, this was the first time that the overproduction of the C50 carotenoids decaprenoxanthin and C.p. 450 in a milligram per gram DW range by a microbial host was described. Izumori et al. could increase the decaprenoxanthin production of *Aureobacterium* sp. FERM P-18698 by the addition of D-piscose to the culture medium, yielding concentrations of 0.7 mg/g DW (Fukuoka et al. 2004).

The three natural C50 carotenoids, decaprenoxanthin, sarcinaxanthin, and C.p. 450, have also been produced heterologously in *E. coli*. Sarcinaxanthin production in a transgenic *E. coli* was described earlier by the heterologous expression of the *P. ananatis* genes *crtEBI* and the *crtE2YgYh* genes from the marine *M. luteus* isolate Otnes 7, which yielded 2.5 mg/g DW nonglucosylated sarcinaxanthin (Netzer et al. 2010). By using the adjustable Pm/*xylS* promoter system for expression of the biosynthetic pathway genes, it was later shown that the sarcinaxanthin production yield could be varied to any level in *E. coli*, enabling identification of bottlenecks and key catalytic

steps for further increased sarcinaxanthin production levels (Lale et al. 2011). Krubasik et al. (2001) demonstrated decaprenoxanthin production in *E. coli* by introducing the biosynthetic pathway genes from *C. glutamicum*, and Tao et al. (2007) used an analogous strategy by recruiting *Dietzia* sp. CQ4 biosynthetic genes for the heterologous production of β-cyclic C.p. 450 in *E. coli*. Interestingly, a synthetic C50 carotenoid biosynthetic pathway was constructed in which the *M. luteus* γ-cyclic C50 cyclase was substituted with the ε-cyclic C50 cyclase from *C. glutamicum*, resulting in a recombinant strain producing three different C50 carotenoids: sarcinaxanthin, decaprenoxanthin, and the novel asymmetric sarprenoxanthin (Netzer et al. 2010). The latter experiments support observations made elsewhere that heterologous carotenoic enzymes can display new and unexpected catalytic properties and specificities in combination with each other in heterologous hosts (see above).

Hydrophobic compounds like carotenoids show higher water solubility upon glycosylation as shown for zeaxanthin (Hundle et al. 1992). Increased water solubility as a consequence of glycosylation might be beneficial for applications of carotenoids (Dembitsky 2005; Heider et al. 2013). The targeted glycosylation of carotenoids in microbial hosts has rarely been described. Within the scope of the elucidation of the sarcinaxanthin biosynthetic genes, the production of sarcinaxanthin glucosides in *E. coli* cells was accomplished by heterologous expression of the necessary *crt* genes in addition to *crtX* from *M. luteus* (Netzer et al. 2010). From these results, the potential of *E. coli* for producing carotenoid glucosides can be deduced, although the nonglucosylated derivative was still produced as a major carotenoid. No further investigation toward concerted production of carotenoid glucosides by recombinant *E. coli* was performed. Recently, the engineering of *C. glutamicum* toward production of various carotenoids in their glucosylated and nonglucosylated form was reported (Heider et al. 2013). They could show that the expression of the native *crtX* yielded the glucosylated C50 carotenoids, decaprenoxanthin, C.p. 450, and sarcinaxanthin. Glucosylation of the C40 zeaxanthin was also achieved by the heterologous expression of *crtX* from *P. ananatis*, for which an expanded substrate range was additionally revealed.

Outlook

Several carotenoid-producing systems have been engineered as microbial platforms, e.g., *E. coli*, *S. cerevisiae*, *C. glutamicum*, or microalgae, mostly for overproduction of carotenoids known from the plant kingdom. With the exploration of C50 carotenoids as engineering targets, this class of compounds comes within reach of large-scale production and utilization. Though promising, such utilization has so far not extensively been explored, likely also due to the limited availability of these compounds. Moreover, the production of C50 carotenoids in microbial systems opens the possibility to derive norisoprenoids from them, by thermal oxidation, by lipoxygenase activity, or by the co-expression of dioxygenases. Clearly, these will be novel compounds with so far unexplored properties. The outlook to produce glycosylated norisoprenoids is also of interest, since this may promote water solubility of these compounds and may allow higher accumulation in microbial cultures, and which may be used as water-soluble precursors for bio-active norisoprenoids.

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