Chapter 10

Carotenoid Production by *Corynebacterium*: The Workhorse of Industrial Amino Acid Production as Host for Production of a Broad Spectrum of C40 and C50 Carotenoids

Nadja A. Henke, Petra Peters-Wendisch and
Volker F. Wendisch

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Abstract

Corynebacterium glutamicum is used as a workhorse of industrial biotechnology for more than 60 years since its discovery as a natural glutamate producer in the 1950s. Nowadays, L-glutamate and L-lysine are being produced with this GRAS organism in the million-ton scale every year for the food and feed markets, respectively. Sequencing of the genome and establishment of a genetic toolbox boosted metabolic engineering of this host for a broad range of industrially relevant compounds ranging from bulk chemicals to high-value products. Carotenoids, the colourful representatives of terpenoids, are high-value compounds whose bio-based production is on the rise. Since *C. glutamicum* is a natural producer of the rare C50 carotenoid decaprenoxanthin, this organism is well suited to establish terpenoid-overproducing platform strains with the help of metabolic engineering strategies. In this work, the carotenogenic background of *C. glutamicum* and the metabolic engineering strategies for the generation of carotenoid-overproducing strains are depicted.

Keywords: *Corynebacterium*, C40/C50 carotenoids, biotechnological production, metabolic engineering, decaprenoxanthin, β-carotene, astaxanthin

1. Introduction

Carotenoids are the dominant pigments for the colouration of food, feed and beverages. The annual demand of the feed additive astaxanthin, for example, is estimated to be 130 tons for

aquaculture and poultry breeding [1]. Besides their yellow-to-red colouring properties, this group of terpenoids is drawing attention in the healthcare industry due to their high antioxidant activities. Since the demand of naturally produced carotenoids is rising, and the fact that extraction of these high-value compounds from plant material is rather cost-inefficient, alternative and flexible production systems are favoured [2].

Corynebacterium glutamicum is a workhorse for the million-ton-scale production of L-glutamate and L-lysine. As a natural carotenoid producer, this biotechnological established microorganism is in focus as a suitable cell factory for natural carotenoid production. The biotechnological application of this genetically accessible bacterium has been exploited for the production of various natural and non-native products [3], including its potential to produce a range of industrially relevant carotenoids.

2. Carotenoid production with *Corynebacterium glutamicum*

2.1. *Corynebacterium glutamicum* as an established cell factory

For more than 60 years, *C. glutamicum* is used as a cell factory for the production of amino acids. Because of its GRAS status, this microbe has a long history in the food and feed industry in the million-ton scale [4]. Since its discovery as a natural glutamate producer in the 1950s [4], its production spectrum was broadened. Lysine is the second biggest production volume being generated by this microbe [5, 6]. Sequencing of its genome [7], the development of a well-filled genetic toolbox [8] and establishment of large-scale fermentations [9] boosted investigations of a wide set of industrially relevant compounds.

This cell factory can naturally utilise glucose, fructose, sucrose, mannitol, arabitol, propionate and acetate under aerobic conditions [10, 11]. Moreover, metabolic engineering enabled utilisation of alternative carbon and energy sources such as glycerol [12], amino sugars [13, 14], β-glucans [15], levoglucosan [16], pentoses [17] and starch [18]. Thus, for industrial fermentations usage of carbon sources which are available in high quantities at low prices is possible, while competition with food and feed resources can be avoided.

Although *C. glutamicum* was discovered as a natural glutamate producer, nowadays, several metabolically engineered production strains are available. Besides the proteinogenic amino acids, also non-proteinogenic amino acid like gamma-aminobutyrate (GABA) [19, 20] and citrulline [21], diamines [22, 23], alcohols [24, 25] and organic acids [26, 27] have been produced. Moreover also high-value compounds including the sesquiterpenoid valencene [28] and the C40 carotenoids β-carotene and astaxanthin [29, 30] can be synthesised with this microbe.

The broad product spectrum from bulk compounds, building blocks, food and feed additives and pharmaceutical and bioactive compounds indicates that *C. glutamicum* has developed to a chassis organism of metabolic engineering. Therefore, several efforts for systematic reduction and optimisation of the *C. glutamicum* genome have been made aiming on a reduced metabolic complexity and strength for future purposes of genetic engineering of new routes for new products [31, 32].

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2.2. Carotenogenesis in *Corynebacterium glutamicum*

C. glutamicum is a yellow-pigmented soil bacterium due to the accumulation of a rare C50 carotenoid decaprenoxanthin and its glucosides [33]. Long-chain C50 carotenoids have been mainly isolated from extremely halophilic archaea [34, 35] and Gram-positive bacteria of the order *Actinomycetales* [36]. Among these species few other corynebacteria are known to produce carotenoid pigments such as *C. michiganense* [37], *C. erythrogenes* [38], *C. fascians* [39] and *C. poinsettia* [40]. Nevertheless, the respective pathways are yet poorly understood due to a lack of complete genome information. Carotenogenesis in *C. glutamicum* has been functionally characterized. The genome of *C. glutamicum* possesses two *crt* operons [7], whereas one of them encodes for all the necessary enzymes responsible for the conversion of the C5 precursor molecules dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) to the C50 carotenoid decaprenoxanthin [41].

2.2.1. Organisation of carotenogenic genes within the chromosome

The precursor molecules derive from the methylerythritol phosphate (MEP) pathway, whose respective genes are partially clustered within the chromosome (**Figure 1**) [30]. The genes *dxs* (cg2083), *ispH* (cg1164) and *idi* (cg2531) are monocistronic, while *dxr* (cg2208), *ispD* (cg2945), *ispE* (cg1039), *ispF* (cg2944) and *ispG* (cg2206) are organised in putative operons. *IspE* is the third gene of the operon cg1037-*ksgA*-*ispE*-cg1040-*pdxK* with genes for a putative resuscitation-promoting factor (cg1037), putative dimethyl adenosine transferase (KsgA) and putative pyridoxamine kinase (PdxK). The genes *ispD* and *ispF* are encoded in the cg2946-*ispDF* operon with cg2946, which codes for a putative CarD-like transcriptional regulator. The genes *dxr* and *ispG* are organised in a transcriptional unit separated by an uncharacterised gene (cg2207) putatively encoding a membrane-embedded Zn-dependent protease. In *C. glutamicum* two prenyltransferases have been characterised [42] which both yield GGPP. Interestingly, the major prenyltransferase IdsA is not encoded within the major *crt* operon [42] but annotated in an operon that is also containing $\alpha(1\rightarrow6)$ mannopyranosyltransferase (**Figure 1**) [43]. CrtE is the second functional GGPP synthase, which is the first gene of the major *crt* operon (**Figure 1**). Both *crt* operons contain a *crtB* gene; however, the one from the small *crt* operon is weakly expressed [41]. Although both *crt* operons contain gene coding for a phytoene desaturase, only CrtI from the major operon is functional [41]. In addition, *crtEb*, $crtY_e$ and $crtY_f$ are part of the major *crt* operon which is necessary for decaprenoxanthin formation (**Figure 4**). Glucosyltransferase gene *crtX* is located in proximity of the major *crt* operon.

Although little is known about carotenogenesis in other corynebacteria, the genomic organisation of corresponding *crt* genes seems to be conserved [41].

2.2.2. Biosynthesis of decaprenoxanthin

The terpenoid precursor molecules DMAPP and IPP derive from the MEP pathway that uses pyruvate and GAP as substrates from central metabolism in *C. glutamicum*. Eight genes are encoding the enzymes which are necessary to build up the C5 precursors under consumption of NADPH, ATP and CTP (**Figure 2**). It has to be mentioned that DXP, the first intermediate

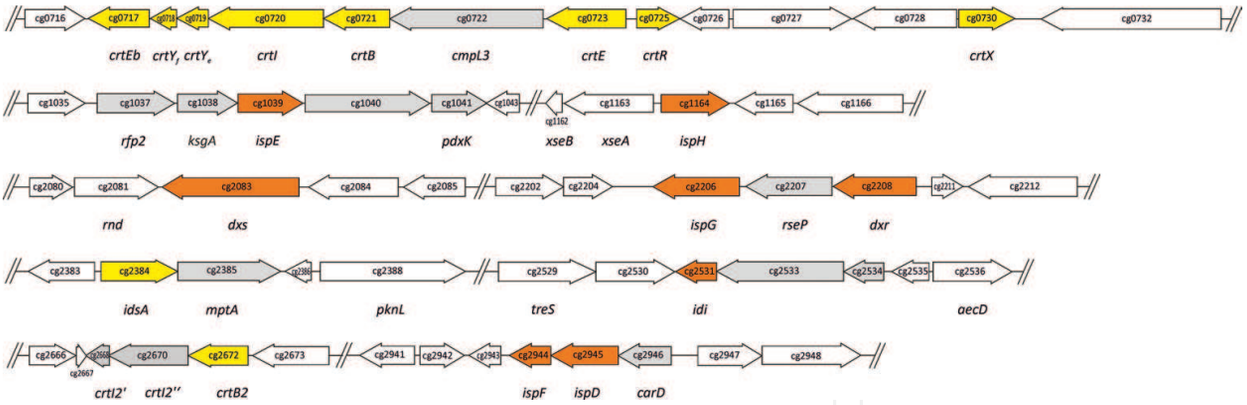

Figure 1. Genomic organisation of carotenogenic genes of *Corynebacterium glutamicum*. The MEP pathway genes involved in the synthesis of the isoprenoid precursors IPP and DMAPP from pyruvate and GAP are depicted in yellow; genes encoding for the decaprenoxanthin biosynthesis pathway from IPP and DMAPP are depicted in orange. Genes that are organised in operons with the respective MEP pathway or decaprenoxanthin pathway genes are shown in grey. Gene names, IDs and respective protein products: *crtEb* (cg0717), lycopene elongase; $crtY_f$ (cg0718) and $crtY_e$ (cg0719), hetero-dimeric C50 ε-cyclase; *crtI* (cg0720), phytoene desaturase; *crtB* (cg0721), phytoene synthase; *cmpL3* (cg0722) RND transporter, corynebacterial membrane protein [44]; *crtE* (cg0723), GGPP synthase; *crtR* (cg0725), transcriptional regulator of carotenoid biosynthesis; *crtX* (cg0730), carotenoid glycosyltransferase; *rfp2* (cg1037), resuscitation-promoting factor 2 [45]; *ksgA* (cg1038), putative 16S ribosomal RNA methyltransferase; *ispE* (cg1039), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; *pdxK* (cg1041), putative pyridoxamine kinase; *xseB* (cg1162), putative exodeoxyribonuclease VII small subunit; *xseA* (cg1163), putative exodeoxyribonuclease VII large subunit; *ispH* (cg1164), 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase; *rnd* (cg2081), putative ribonuclease D; *dxs* (cg2083), 1-deoxy-D-xylulose-5-phosphate synthase; *ispG* (cg2206), (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase; *rseP* (cg2207), putative membrane-embedded Zn-dependent protease; *dxr* (cg2208), 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *idsA* (cg2384), GGPP synthase; *mptA* (cg2385), α(1→6) mannopyranosyltransferase [43]; *treS* (cg2529), trehalose synthase [46]; *idi* (cg2531), isopentenyl-diphosphate δ-isomerase; *aecD* (cg2536), cystathionine β-lyase [47]; *pknL* (cg2388), serine/threonine protein kinase [48]; *ispF* (cg2944), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *ispD* (cg2945), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase and *carD* (cg2946), putative CarD-like transcriptional regulator.

of the MEP pathway, is also a precursor molecule for thiamine biosynthesis in *C. glutamicum*. Two prenyltransferases IdsA and CrtE use one molecule DMAPP and three molecules IPP to form GGPP (C20) (**Figure 2**) [41]. Two GGPP molecules can be condensed by a phytoene synthase CrtB to phytoene, a colourless C40 structure (**Figure 2**). The red lycopene is formed via the phytoene desaturase CrtI [17].

Lycopene is a central metabolite for all C40 and C50 carotenoids (**Figures 3** and **4**). In *C. glutamicum* this C40 carotenoid is further elongated by the lycopene elongase CrtEb with two DMAPP molecules to form the linear C50 carotenoid flavuxanthin. Finally, a heterodimeric ε-cyclase $CrtY_eY_f$ introduces ε-cyclic moieties [49] in that linear C50 structure to form decaprenoxanthin (**Figure 4**). Decaprenoxanthin can be glycosylated either at one or at both terminal hydroxy groups by CrtX [50]. The physiological function remains unknown; however, glycosylation of end groups generally yields a more polar molecule structure in comparison to the free decaprenoxanthin and might change integrity into the membrane.

2.2.3. The unusual cell envelope of C. glutamicum and carotenoid accumulation

The cell envelope of the Gram-positive bacterium *C. glutamicum* possesses a special and complex cell wall structure [51, 52]. Besides the inner plasma membrane, the peptidoglycan and

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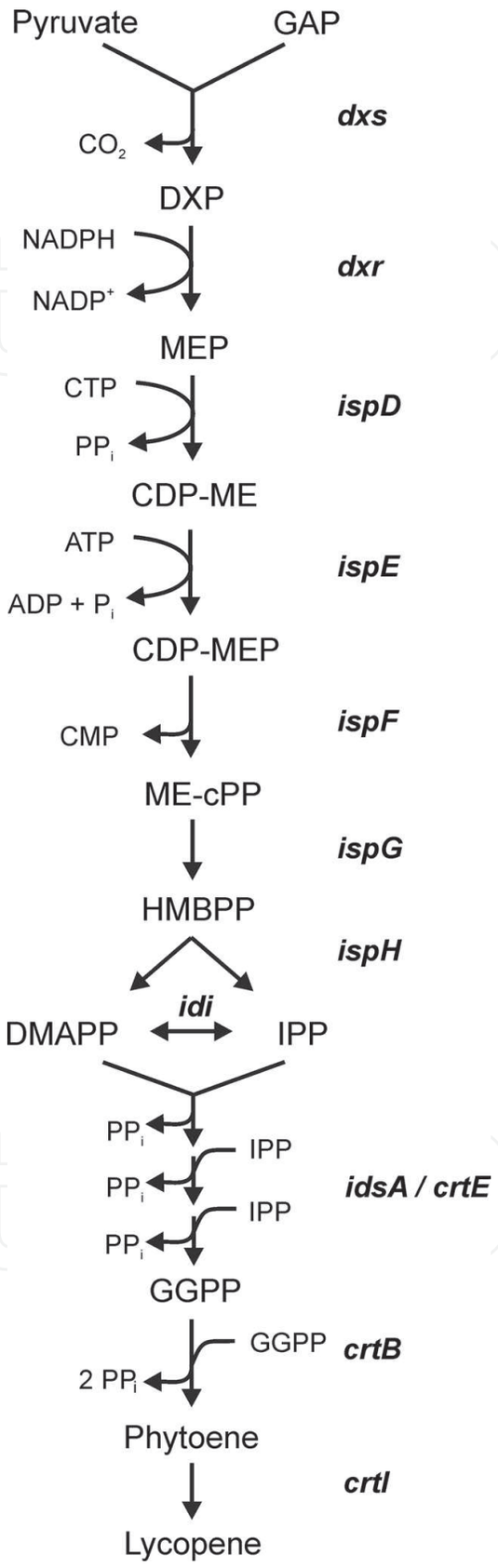

Figure 2. Metabolic pathway of lycopene production in *Corynebacterium glutamicum* starting from GAP and pyruvate. Genes are shown next to the reaction catalysed by the encoded enzyme (see **Figure 1**; *crtE/idsA*: prenyltransferase; *crtB*: phytoene synthase and *crtI*: phytoene desaturase).

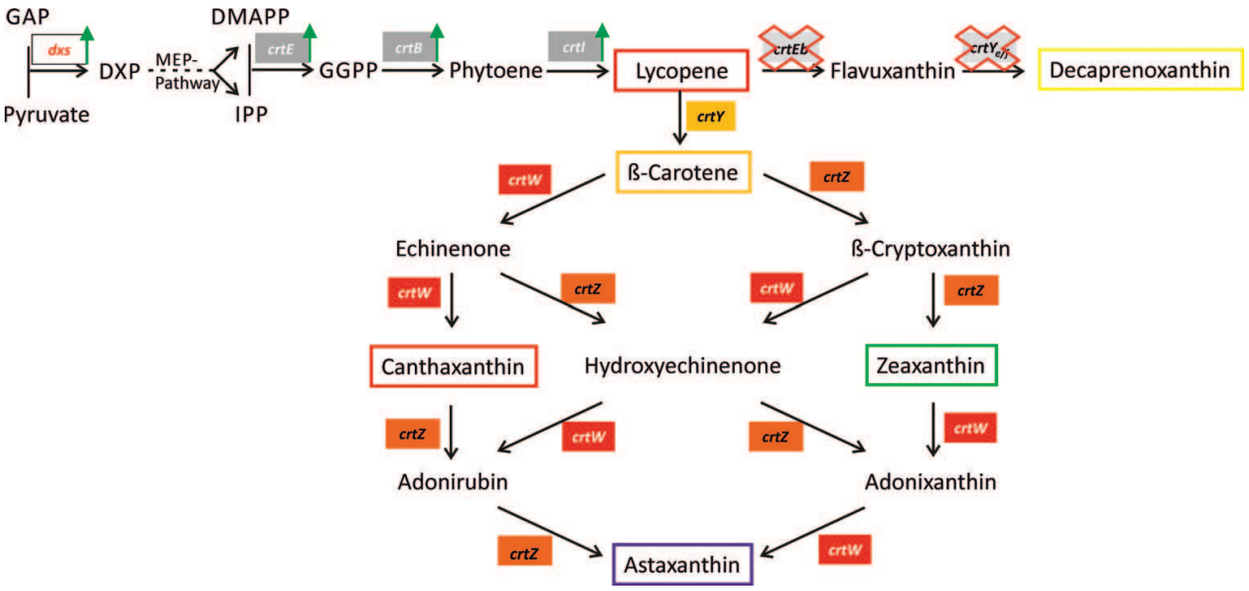

Figure 3. Metabolically engineered platform strains for production of cyclic C40 carotenoids. The biosynthesis of C40 carotenoids from the precursor molecules dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) is depicted. Genes are shown next to the reaction catalysed by the encoded enzyme (*crtE*: GGPP synthase; *crtB*: phytoene synthase; *crtI*: phytoene desaturase; *crtEb*: lycopene elongase; *crtYe/f*: C45/50 carotenoid ε-cyclase; *crtY*: lycopene cyclase; *crtZ*: β-carotene hydroxylase and *crtW*: β-carotene ketolase). Endogenous genes are highlighted in grey boxes and overexpressions indicated by green arrows. Heterologous genes are highlighted in coloured boxes.

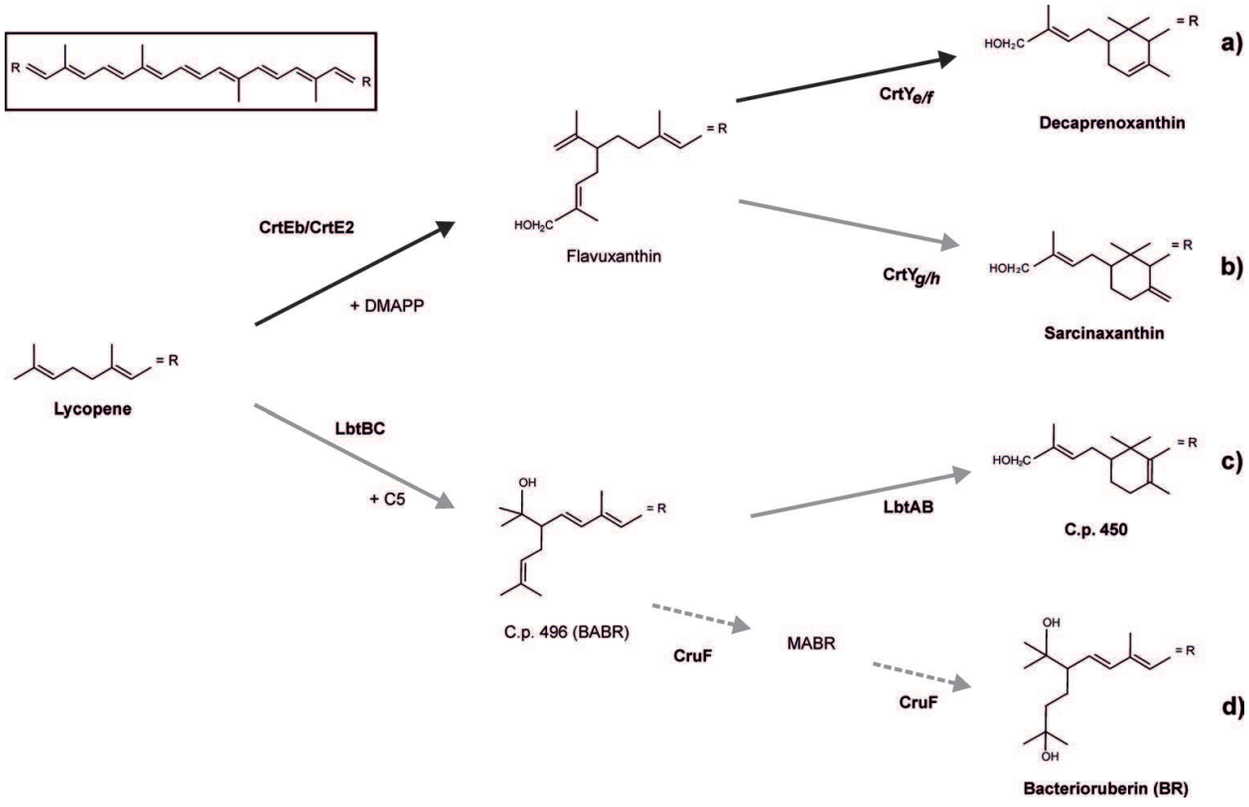

Figure 4. Biosynthesis of C50 carotenoids starting from the precursor lycopene. The native pathway of decaprenoxanthin biosynthesis (a) is shown with black arrows. Established non-native biosynthesis pathways of sarcinaxanthin (b) and C.p. 450 (c) are indicated by grey arrows. Possible biosynthesis of bacterioruberin (d) from C.p. 496 (BABR) is indicated by dashed grey arrows.

arabinogalactan layer forms a polysaccharide barrier that is esterified to mycolic acids. The presence of the mycolic acid layer is a phylogenetic trait of *Corynebacterianeae* [52]. The plasma membrane consists of polar lipids, mainly phospholipids which form together with proteins the typical lipid bilayer.

Carotenoids are usually attached to or span membranes due to their lipophilic character and rigid structure. Association of carotenoids to membranes often results in a decreased water permeability and increased firmness, thus supporting membrane stability [53, 54]. It is hypothesized that this is closely linked to their function supporting resistances to osmotic stresses, heat or radiation [54–56]. Moreover, it was shown that incorporation of carotenoids into a membrane is more efficient when the carbon backbone length of the carotenoid correlates with the thickness of the phospholipid bilayer [57]. Although decaprenoxanthin is a C50 carotenoid, it is assumed to be integrated into the plasma membrane [58, 59] as it was also shown for most C40 carotenoids of other bacteria [53, 56, 60].

2.3. Metabolic engineering of *Corynebacterium glutamicum* for carotenoid production

Since *C. glutamicum* naturally produces a rare cyclic C50 carotenoid, its potential to produce industrially relevant C40 carotenoids was elucidated over the last years in more detail. First, it was shown that production of both non-native C50 and C40 carotenoids was possible with this production host [50, 61]. Secondly, improvement of the MEP pathway yielded enhanced production [30]. Finally, the production of industrially relevant cyclic C40 carotenoids was shown on the basis of balancing of the enzyme quantities and on the basis of a screen for suitable enzymes for enhanced production [29].

2.3.1. Design of a platform strain for the production of the central intermediate lycopene

For production of non-native C40 and C50 carotenoids, endogenous decaprenoxanthin production has to be avoided (**Figure 3**). For this reason, a prophage-cured *C. glutamicum* strain MB001 [62] was metabolically engineered by deleting the genes *crtEb* and $crtY_eY_f$, resulting in the biosynthesis of the central intermediate lycopene [41, 50]. The supply of the precursor molecule DMAPP and its isomer IPP was successfully engineered through improved expression of *dxs*, encoding for the first committed step in the MEP pathway, on the basis of a chromosomal promoter exchange [30]. Furthermore, it was shown that overproduction of the prenyltransferase (CrtE), phytoene synthase (CrtB) and phytoene desaturase (CrtI) strongly enhanced lycopene production [50]. Chromosomal integration of the artificial operon *crtEBI* under the control of a strong constitutive *tuf* promoter in a $\Delta crtEbY_eY_f$ strain yielded a red phenotype [29].

2.3.2. Metabolic engineering for cyclic C40 carotenoid productions

On the basis of lycopene-producing platform strains, a collection of cyclic C40 carotenoids could be generated via different metabolic engineering strategies. First of all, β-cyclisation of lycopene was accomplished via heterologous overexpression of *crtY* from *Pantoea ananatis* [29, 30]. Here, either plasmid-driven or genome-based expressions revealed complete conversion of lycopene to β-carotene (**Figure 3**). This might rely on the fact that β-carotene has a more polar structure due to its β-ionone rings and thus integrates more efficiently in the phospholipid bilayer

than lycopene. Heterologous expression of β-carotene hydroxylase from *P. ananatis* in such a β-carotene–accumulating strain yielded production of zeaxanthin [30]. Additional plasmid-driven overproduction of a β-carotene ketolase from *Brevundimonas aurantiaca* showed accumulation of the red C40 carotenoid astaxanthin for the first time in *C. glutamicum* [30]. Analysis of the carotenoid profile showed that conversion of β-carotene to its oxygenated derivatives was not efficient maybe due to an unbalanced expression of involved genes.

Pathway balancing was performed through balancing of the enzyme levels of β-carotene ketolase and hydroxylase (**Figure 3**). In a combinatorial approach, enzyme quantities were varied on the basis of varied translation initiation rates [29]. The corresponding genes were assembled in an expression vector under the strong constitutive *tuf* promoter using different ribosome-binding sites, different spacing lengths and different start codons. The translation initiation rate is depending on the free binding energy of the ribosome-binding site and the 16S rRNA as well as on the free energy of secondary structures of the mRNA [29]. Secondary structures between 5′UTR and coding region are depending on the expressed gene and were calculated with the RBS calculator [63]. Analysis revealed that the higher the TIRs of both genes, the higher the astaxanthin titer [29] in recombinant *C. glutamicum*. With this approach an astaxanthin content of 0.3 mg/g CDW was accomplished indicating that aside pathway balancing also enzyme activities or stabilities could be limiting the production.

The analysis of carotenogenesis identified a transcriptional regulator CrtR [64]. This MarR regulator is binding to the promoter sequence of *crtE* and thus inhibits transcription of the carotenogenic genes. Deletion of the corresponding gene located divergent to the major *crt* operon enhanced both native and non-native carotenoid productions. The production titers of non-native C50 carotenoids sarcinaxanthin and C.p. 450 improved 1.5 and 2-fold, respectively. For the deregulated β-carotene–accumulating strain production, titer doubled to approximately 12 mg/g CDW [29]. This platform strain was used for a second approach to produce astaxanthin with recombinant *C. glutamicum.* In the deregulated β-carotene–producing platform strain, a screening of potential β-carotene ketolases and hydroxylases of rather uncharacterised gene donors for production of canthaxanthin and zeaxanthin was done. Two enzymes from *Fulvimarina pelagi* showed the best conversion of β-carotene to the oxygenated derivatives [29]. Moreover, a combination of those two genes with a two-vector system resulted in a *C. glutamicum* strain producing 1.6 mg/g CDW of astaxanthin [29], which is the highest reported titer obtained with this organism. Since *C. glutamicum* is fast growing even in shake-flask experiments, the volumetric productivity of 0.4 mg L^{-1} h^{-1} is competitive to algal and yeast-based productions.

2.3.3. Heterologous gene expression for production of C50 carotenoids

On the basis of lycopene-accumulating platform strains, production of a range of C50 carotenoids was established (**Figure 4**). The cyclic C50 carotenoids sarcinaxanthin and C.p. 450 can be derived from lycopene via the heterologous expression of corresponding lycopene elongase and linear C50 carotenoid cyclases. For sarcinaxanthin production genes from the *Micrococcus luteus* [36] were cloned and expressed from a plasmid. The lycopene elongase CrtE2 from this organism adds two DMAPP molecules at C2 and C2′ positions which is identical with the catalytic activity of *C. glutamicum* endogenous lycopene elongase. Simultaneously hydroxylation

at the C1/C1′ positions is postulated to stabilise the prenylated carbocation. The linear C50 carotenoid flavuxanthin is cyclised to sarcinaxanthin by a heterodimeric γ-cyclase $CrtY_gY_h$ [36]. This cyclase acts exclusively on a linear C50 backbone and not one lycopene as it holds also true for the ε-cyclase from *C. glutamicum* [36, 49]. Production of C.p. 450 was entailed through plasmid-driven heterologous expression of genes from *Dietzia* sp. CQ4 [65]. Here, elongation of lycopene by LbtBC results in a slightly different linear C50 carotenoid, C.p. 496. Cyclisation by the heterodimeric β-cyclase LbtAB yields C.p. 450. Moreover, starting from C.p. 496, also heterologous synthesis of bacterioruberin could be possible by expression of *cruF*, e.g. from *Haloarcula japonica* [66]; however, this route is not yet established.

2.4. Future prospects: advancing rational strain engineering for short- and long-chain terpenoid production

Based on the findings of the research on carotenoid biosynthesis in *C. glutamicum*, new strategies for rational strain engineering on terpenoid productions are now available. First, deletion of endogenous carotenogenic genes will yield accumulation of the terpenoid precursor molecules DMAPP and IPP in a prophage-cured *C. glutamicum* MB001. Secondly, the application of carbon-chain length-specific prenyltransferases will either yield GPP, FPP or GGPP, allowing their conversion to mono-, di-, sesqui- or polyterpenoids. Besides the C40 and C50 carotenoids, it was also shown that *C. glutamicum* is a suitable host for production of short-chain terpenoids like valencene [28]. Valencene is a sesquiterpene which is derived from FPP. It was shown that heterologous expression of *ispA* from *Escherichia coli* yielded the precursor FPP instead of GGPP as synthesised by native prenyltransferases IdsA and CrtE. Therefore, engineering combinations of native, mutated or heterologous prenyltransferases with terpene synthases from other bacteria or plants are the keys to broaden the terpenoid product spectrum of metabolically engineered *C. glutamicum*.

Since many short-chain terpenoids exhibit antimicrobial properties, timed induction of terpene synthase gene expression is a strategy to face this challenge. An optogenetic approach using photolabile caged IPTG as inducer was successfully applied to allow (i) altered expression levels and (ii) non-invasive timed induction of heterologous genes in a valencene-producing *C. glutamicum* strain [67]. Moreover, recent findings have proven that general transcription machinery engineering (gTME) is an efficient approach to improve carotenoid production in *C. glutamicum* [68]. In this study, carotenoid production was improved in the stationary growth phase either by overexpression of primary sigma factor gene *sigA* or by deletion of alternative sigma factor gene *sigB* [68]. Biosensors have been used in *C. glutamicum* for efficient screening of mutant libraries to find novel targets for metabolic engineering and for positive or negative on-demand control of metabolic pathways or enzymes [64]. An application of a biosensor was already successfully implemented for screening L-lysine-producing *C. glutamicum* strains [69]. Basically, a transcriptional fusion of a regulated promoter and a reporter gene in the presence of the corresponding transcriptional regulator (LysG) enabled intracellular metabolite (lysine) sensing and isolation of lysine-accumulating mutants. On the other hand, intracellular riboswitch-based L-lysine biosensors have been developed to induce the gene of the lysine export system or to repress the citrate synthase gene resulting in increased lysine production [70, 71]. Recently, the CRISPRi/dCas9 system has been estab-

lished for efficient downregulation of target genes in *C. glutamicum* as exemplified for lysine and glutamate production targets [72]. In summary, a foundation has been laid for employing *C. glutamicum* as a natural terpenoid producer and the recent progress in method and tool development for rational engineering of this biotechnological workhorse foreshadow exciting options for terpenoid production processes using this bacterium.

Author details

Nadja A. Henke, Petra Peters-Wendisch and Volker F. Wendisch*

*Address all correspondence to: volker.wendisch@uni-bielefeld.de

Genetics of Prokaryotes, Faculty of Biology & CeBiTec, Bielefeld University, Germany

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