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Production and glucosylation of C_{50} and C_{40} carotenoids by metabolically engineered *Corynebacterium glutamicum*

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Abstract The yellow-pigmented soil bacterium *Corynebacterium glutamicum* ATCC13032 is accumulating the cyclic C_{50} carotenoid decaprenoxanthin and its glucosides. Carotenoid pathway engineering was previously shown to allow for efficient lycopene production. Here, engineering of *C. glutamicum* for production of endogenous decaprenoxanthin as well as of the heterologous C_{50} carotenoids C.p.450 and sarcinaxanthin is described. Plasmid-borne overexpression of genes for lycopene cyclization and hydroxylation from *C. glutamicum*, *Dietzia* sp., and *Micrococcus luteus*, in a lycopene-producing platform strain constructed here, resulted in accumulation of these three C_{50} carotenoids to concentrations of about 3–4 mg/g CDW. Chromosomal deletion of a putative carotenoid glycosyltransferase gene cg0730/*crtX* in these strains entailed production of non-glucosylated derivatives of decaprenoxanthin, C.p.450, and sarcinaxanthin, respectively. Upon introduction of glucosyltransferase genes from *M. luteus*, *C. glutamicum*, and *Pantoea ananatis*, these hydroxylated C_{50} carotenoids were glucosylated. We here also demonstrate production of the C_{40} carotenoids β-carotene and zeaxanthin in recombinant *C. glutamicum* strains and co-expression of the *P. ananatis crtX* gene was used to obtain glucosylated zeaxanthin. Together, our results show that *C. glutamicum* is a potentially valuable host for production of a wide range of glucosylated C_{40} and C_{50} carotenoids.

Keywords *Corynebacterium glutamicum* · C_{50} carotenoid production · *crtX* · Glucosyltransferase · Decaprenoxanthin · Sarcinaxanthin · Lycopene · ß-carotene · Zeaxanthin

Introduction

Carotenoids are natural yellow- to red-colored pigments synthesized by plants, fungi, algae, and bacteria (Britton et al. 2004). They can have diverse biological functions such as photoprotection or light-harvesting molecules or as membrane stabilizers (Lee and Schmidt-Dannert 2002; Sandoval et al. 1984). Animals must obtain carotenoids through their diet and some have important functions such as precursors for vitamin A (Johnson and Schroeder 1996) and different hormones (Vershinin 1999). Commercially, carotenoids are used as food colorants, animal feed supplements, and cosmetics as well as in the neutraceutical and pharmaceutical industries due to their health benefits (Lee and Schmidt-Dannert 2002; Ye and Bhatia 2012). The total value of carotenoids was reported as $1.2 billion in 2010 with a yearly growth of 2.3 % on the global market (Cutzu et al. 2013). In spite of the impressive number of already identified microbial carotenoids, only a few are produced at the industrial scale and the interest in efficient production of carotenoids by microbial hosts is growing (Cutzu et al. 2013).

Carotenoids originate from the terpenoid pathway and are derived from the universal precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMPP) (Rodriguez-Villalon et al. 2008). More than 95 % of all carotenoids are based on the symmetric C_{40} backbone phytoene and only a rare number of C_{30} and C_{50} carotenoids have been described (Tobias and Arnold 2006). C_{50}

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carotenoids are formed by a diprenylation step (addition of two C5 units) and hydroxylation of C_{40} carotenoids. C_{50} carotenoids have multiple conjugated double bounds, and they contain at least one hydroxyl group; both these features contribute to strong antioxidative properties (Netzer et al. 2010). The different number of conjugated double bonds shifts their light absorption and reactive oxygen quenching properties (Osawa et al. 2010; Jackson et al. 2008; Miller et al. 1996; Naguib 2000). Therefore, the C_{50} carotenoids have high potential for application in sunscreens and other light-protecting cosmetics as it was described to be an effective UV and visible light filter. Their antioxidative properties, golden yellow color, solubility, and stability in oil emulsions add to the attractiveness for the usage in cosmetic products [US 2013/0078203 A1].

C_{50} carotenoids occur only in a restricted number of bacterial species and have so far only been found in certain Gram-positive bacteria of the order *Actinomycetales* (Netzer et al. 2010) in addition to a Gram-negative *Pseudomonas* strain (Miki et al. 1994).

Corynebacterium glutamicum is a known producer of the C_{50} carotenoid decaprenoxanthin and its glucosylated forms (Krubasik et al. 2001b), and is the workhorse for large-scale amino acid production. In 2012, the fermentative production of L-glutamate and L-lysine amounted to about 2,930,000 and 1,950,000 tons, respectively (Ajinomoto, Inc., available http://www.ajinomoto.com/en/ir/pdf/FY13Q1_data_E.pdf, Cited 05 September 2013). The genetics, metabolism, and physiology of *C. glutamicum* have been extensively investigated; it has a well-established genetic tool box, and relaxed growth requirements. Therefore, this bacterium should be a potentially promising candidate host for the industrial production of carotenoids.

To date, biosynthesis of three different C_{50} carotenoids initiating with lycopene have been described: the biosynthetic pathways of the ε-cyclic C_{50} carotenoid decaprenoxanthin in *C. glutamicum* (Krubasik et al. 2001a; Krubasik and Sandmann 2000), the β-cyclic C_{50} carotenoid C.p.450 in *Dietzia* sp. CQ4 (Tao et al. 2007), and the γ-cyclic C_{50} carotenoid sarcinaxanthin in *Micrococcus luteus* NCTC2665 (Netzer et al. 2010) (Fig. 1). In all three organisms, the respective carotenogenic genes (*crt*) are clustered. In the case of *C. glutamicum*, we recently showed that the six carotenogenic genes (together with a gene of unknown function, cg0722) are forming a transcription unit (Heider et al. 2012). *C. glutamicum* possesses a certain degree of redundancy in the biosynthesis of decaprenoxanthin as it possesses a second functional phytoene synthase gene *crtB2* that is encoded in a small operon comprising *crtB2*, cg2670, and cg2668 (Heider et al. 2012).

After formation of lycopene, the decaprenoxanthin biosynthesis proceeds via a diprenylation catalyzed by the *crtEb* gene product lycopene elongase yielding the acyclic C_{50} carotenoid flavuxanthin. The following cyclization of flavuxanthin to decaprenoxanthin is catalyzed by heterodimeric carotenoid ε-cyclase, encoded by $crtY_e$ and $crtY_f$ (Eggeling and Bott 2005; Krubasik et al. 2001b; Netzer et al. 2010; Sandmann and Yukawa 2005). Besides *C. glutamicum*, a few other *Corynebacteria* were supposed to possess *crt* genes in their genomes, e.g., *Corynebacterium efficiens* YS-314 with a gene cluster comparable to the one in *C. glutamicum* WT (Heider et al. 2012). Details on carotenoid pigments are unknown for these *Corynebacterium* species, whereas *Corynebacterium michiganense* (Saperstein and Starr 1954), *Corynebacterium erythrogenes* (Hodgkiss et al. 1954), *Corynebacterium fascians* (Prebble 1962), and *Corynebacterium poinsettiae* (Starr and Saperstein 1953) are known to contain carotenoid pigments.

Even though the abundance of mono- and diglucosylated decaprenoxanthin in *C. glutamicum* is known for many years, the genes and enzymes for glucosylation of decaprenoxanthin have not been described so far (Netzer et al. 2010). In *M. luteus*, the gene *crtX*, which is located in the *crt*-gene cluster, was shown to encode an enzyme active as sarcinaxanthin glucosyltransferase (GT) (Netzer et al. 2010). CrtX from *M. luteus* shares 46 % identity in the amino acid sequence with the putative GT from *Dietzia* sp. CQ4 (Netzer et al. 2010; Tao et al. 2007). The protein product of cg0730 of *C. glutamicum* shows 35 and 33 % primary sequence identity to CrtX proteins of *M. luteus* and *Dietzia* sp. CQ4, respectively.

In this study, the potential of *C. glutamicum* as a production strain for lycopene-derived terpenoids was investigated by genetic engineering of the carotenoid biosynthetic pathway and we demonstrate that this bacterium can produce a range of different C_{50} and C_{40} carotenoids. We also identified a gene (*crtX*) encoding a putative carotenoid GT in *C. glutamicum* and demonstrated that the encoded protein glucosylated decaprenoxanthin, the C_{50} carotenoids sarcinaxanthin and C.p.450, but not the C_{40} carotenoid zeaxanthin. Production of glucosylated zeaxanthin was achieved by expression of the C_{30}/C_{40} carotenoid GT CrtX from *Pantoea ananatis*, which further demonstrated the potential of this bacterium for production of different carotenoids. In addition, we showed for the first time that CrtX from *P. ananatis* is also able to glucosylate the C_{50} carotenoid decaprenoxanthin.

Material and methods

Bacterial strains, media, and growth conditions

The strains and plasmids used in this work are listed in Table 1. *C. glutamicum* ATCC13032 was used as wild type (WT). Precultivation of *C. glutamicum* strains was performed in BHI or LB medium. For cultivation in CGXII medium

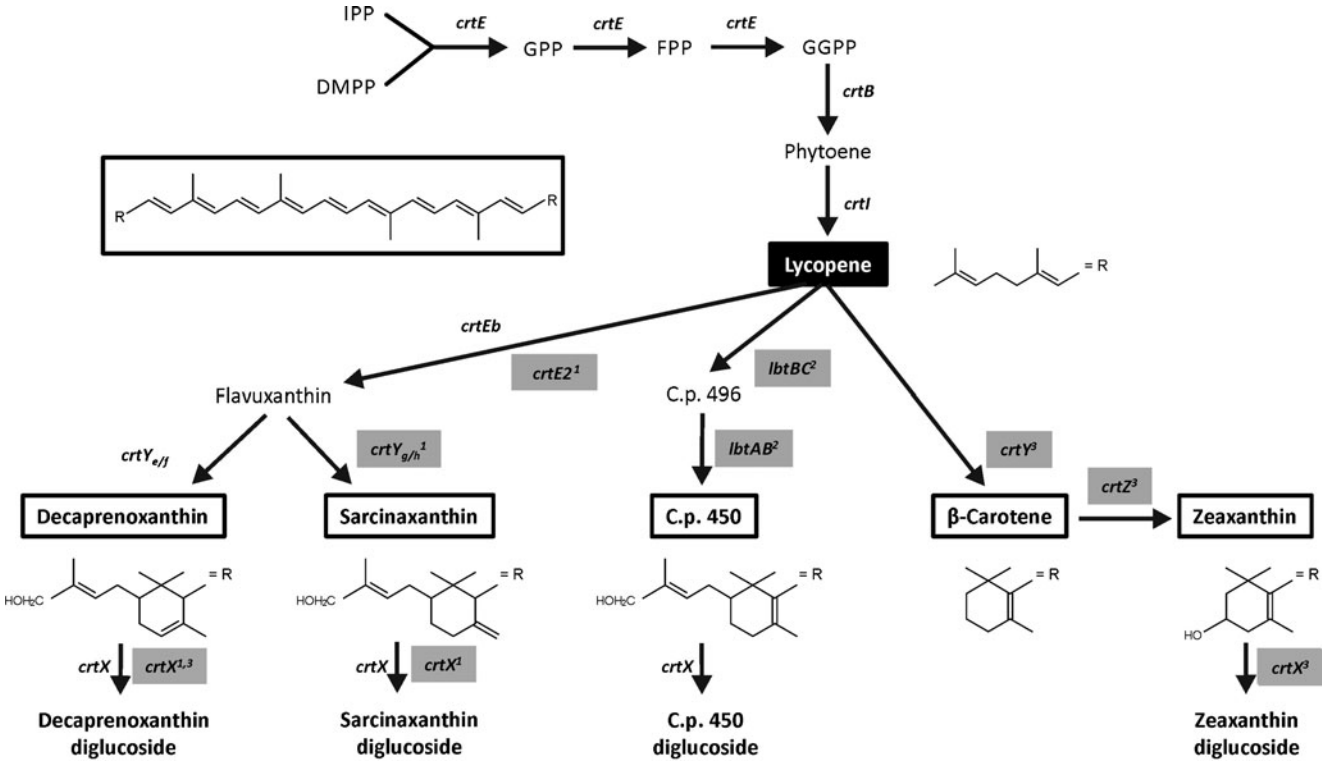

Fig. 1 Schematic representation of the decaprenoxanthin biosynthesis pathway of *C. glutamicum* and of heterologous pathways leading to other carotenoids. The linear carotenoid backbone structure with terminal residues labeled R is shown in a *box* and residues for lycopene, decaprenoxanthin, sarcinaxanthin, C.p.450, β-carotene, and zeaxanthin are depicted *below*. For each enzyme, its gene is given, with heterologously expressed genes originating from *M. luteus* ([1]), *Dietzia* sp. CQ4 ([2]), and *P. ananatis* ([3]) being highlighted in *gray boxes* (for gene names see text). One or both of the terminal hydroxyl groups of the C_{50} carotenoids may be glucosylated by CrtX from *C. glutamicum*, *M. luteus*, and *P. ananatis*, while only CrtX from *P. ananatis* glucosylated zeaxanthin. Abbreviations: *IPP*, isopentenyl pyrophosphate; *DMPP*, dimethylallyl pyrophosphate; *GPP*, geranyl pyrophosphate; *FPP*, farnesyl pyrophosphate; *GGPP*, geranylgeranyl pyrophosphate; *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

(Eggeling and Reyes 2005), precultivated cells were washed once with CGXII medium without carbon source and inoculated to an initial OD_{600} of 1. Glucose was added as carbon and energy source to a concentration of 100 mM. Standard cultivations of *C. glutamicum* were performed at 30 °C in a volume of 50 ml in 500 ml flasks with two baffles shaking at 120 rpm. The OD_{600} was measured in dilutions using a Shimadzu UV-1202 spectrophotometer (Duisburg, Germany). For cloning, *Escherichia coli* DH5α was used as host and cultivated in LB medium at 37 °C. When appropriate, kanamycin or spectinomycin was added to concentrations of 25 and 100 μg/ml, respectively. Gene expression was induced by adding 50 μM and 1 mM IPTG, respectively, at inoculation of the main culture.

Recombinant DNA work

Plasmids were constructed in *E. coli* DH5α from polymerase chain reaction (PCR)-generated fragments (KOD, Novagen, Darmstadt, Germany) and isolated with the QIAprep spin miniprep kit (QIAGEN, Hilden, Germany). Oligonucleotides used in this study were obtained from Metabion GmbH (Martinsried, Germany) and are listed in Table S1. Standard reactions like restriction, ligation, and PCR were performed as described previously (Sambrook and Russell 2001). Besides the common ligation reaction, the Gibson assembly has been applied for the construction of plasmids (Gibson et al. 2009). If applicable, PCR products were purified using the PCR purification kit or MinElute PCR purification kit (QIAGEN, Hilden, Germany). For transformation of *E. coli*, the RbCl method was used (Hanahan 1983) and *C. glutamicum* was transformed via electroporation (van der Rest et al. 1999) at 2.5 kV, 200 Ω, and 25 μF. All cloned DNA fragments were shown to be correct by sequencing.

Construct design and codon optimization of the C_{50} carotenoid synthesis gene cluster of *Dietzia* sp. CQ4

The gene sequences of *lbtA* and *lbtBC* of the *Dietzia* sp. CQ4 were obtained from the NCBI GeneEntrez Database

Table 1 Strains and plasmids used

Strain or plasmid	Relevant characteristics or sequence	Source or reference
Strains		
E. coli DH5α	F⁻ *thi*-1 *endA1 hsdR17*(r⁻ m⁻) *supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *recA1 gyrA96 relA1*	Hanahan (1983)
Pantoea ananatis	ATCC 19321	Misawa et al. (1990)
C. glutamicum strains		
WT	ATCC 13032	Abe et al. (1967)
Δ*crtEb*	*crtEb* deletion mutant of *C. glutamicum* WT	Heider et al. (2012)
Δ*crtYEb*	$crtY_eY_fEb$ deletion mutant of *C. glutamicum* WT	This work
Δ*crtX*	cg0730 (*crtX*) deletion mutant of *C. glutamicum* WT	This work
Δ*crtYEb* Δ*crtX*	Multiple deletion mutant of the genes $crtY_eY_fEb$ and cg0730 of *C. glutamicum* WT	This work
LYC1	Δ*crtYEb* derivative including the expression vector pVWEx1-*crtEBI*	This work
LYC2	Δ*crtYEb* Δ*crtX* derivative including the expression vector pVWEx1-*crtEBI*	This work
DEC1	LYC1 derivative including the expression vector pEKEx3-*crtYEb*	This work
DEC2	LYC1 derivative including the expression vector pEKEx3-*crtEbY*	This work
DEC3	LYC2 derivative including the expression vector pEKEx3-*crtYEb*	This work
SAX1	LYC1 derivative including the expression vector pEKEx3-crtE2Y	This work
SAX2	LYC2 derivative including the expression vector pEKEx3-crtE2Y	This work
CP1	LYC1 derivative including the expression vector pEKEx3-lbtABC	This work
CP2	LYC2 derivative including the expression vector pVWEx1-*crtEBI* and pEKEx3-lbtABC	This work
BCR	LYC2 derivative including the expression vector pEKEx3-$crtY_{Pa}$	This work
ZEA	LYC2 derivative including the expression vector pEKEx3-$crtYZ_{Pa}$	This work
Plasmids		
pK19*mobsacB*	Km^R; *E. coli*/*C. glutamicum* shuttle vector for construction of insertion and deletion mutants in *C. glutamicum* (pK18 $oriV_{Ec}$ *sacB lacZ*α)	Schäfer et al. (1993)
pK19*mobsacB*-Δcg0730	pK19*mobsacB* with a cg0730 deletion construct	This work
pK19*mobsacB*-Δ*crtEb*	pK19*mobsacB* with a *crtEb* deletion construct	Heider et al. (2012)
pK19*mobsacB*-Δ*crtYEb*	pK19*mobsacB* with a $crtY_eY_fEb$ deletion construct	This work
pVWEx1	Km^R; *E. coli*/*C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, $lacI^q$, pCG1 $oriV_{Cg}$)	Peters-Wendisch et al. (2001)
pVWEx1-*crtEBI*	pVWEx1 derivative for IPTG-inducible expression of *crtE*, *crtB* and *crtI* from *C. glutamicum* containing artificial ribosome binding sites in front of *crtI* and *crtBI*	This work
pVWEx1-*crtX*	pVWEx1 derivative for IPTG-inducible expression of cg0730 from *C. glutamicum* containing an artificial ribosome binding site	This work
pEKEx3	$Spec^R$; *E. coli*/*C. glutamicum* shuttle vector for regulated gene expression (P_{tac}, $lacI^q$, pBL1 $oriV_{Cg}$)	Stansen et al. (2005)
pEKEx3-*crtYEb*	pEKEx3 derivative for IPTG-inducible expression of *crtYEb* from *C. glutamicum* containing a single artificial ribosome binding site in front of *crtY*	Heider et al. (2012)
pEKEx3-*crtEbY*	pEKEx3 derivative for IPTG-inducible expression of *crtEb* and *crtY* from *C. glutamicum* containing artificial ribosome binding sites in front of each gene	This work
pEKEx3-$crtE2Y_{Ml}$	pEKEx3 derivative for IPTG-inducible expression of *crtE2 and crtYg/h* from *M. luteus* containing a single artificial ribosome binding site in front of *crtE2*	This work
pEKEx3-*lbtABC*	pEKEx3 derivative for IPTG-inducible expression of the codon optimized *lbtA and lbtBC* from *Dietzia* sp. CQ4 containing artificial ribosome binding sites in front of each gene	This work
pEKEx3-$crtX_{Cg}$	pEKEx3 derivative for IPTG-inducible expression of cg0730 from *C. glutamicum* containing an artificial ribosome binding site	This work
pVWEx1-$crtX_{Ml}$	pVWEx1 derivative for IPTG-inducible expression of crtX from *M. luteus* containing an artificial ribosome binding site	This work
pVWEx1-$crtX_{Pa}$	pVWEx1 derivative for IPTG-inducible expression of cg0730 from *P. ananatis* containing an artificial ribosome binding site	This work
pEKEx3-$crtY_{Pa}$	pEKEx3 derivative for IPTG-inducible expression of *crtY* from *P. ananatis* containing an artificial ribosome binding site	This work
pEKEx3-*crtYZ*	pEKEx3 derivative for IPTG-inducible expression of *crtY* and *crtZ* from *P. ananatis* containing artificial ribosome binding sites in front of each gene	This work
pCRT-E2YgYh-O7	Plasmid for expression of *crtE2* and $crtY_{g/h}$ of *Micrococcus luteus* isolate Otnes 7	Netzer et al. (2010)

Table 1 (continued)

Strain or plasmid	Relevant characteristics or sequence	Source or reference
pMK-RQ8-*lbtA*-*lbtBC*	*E. coli* expression vector including the synthesized and codon optimized genes *lbtA* and *lbtBC* originally from *Dietzia* sp. CQ4	Life Technologies

(GenBank: DQ369754.1). The coding regions were optimized using the GeneOptimizer® expert software (Raab et al. 2010) and synthesized by GeneArt® life technologies™ (Darmstadt, Germany) and the sequence was submitted to GenBank with accession number KF693237. An artificial ribosome binding site and a spacer sequence was inserted in front of each coding sequence. Apart from that, the sequence was flanked by 20 bp, homologous to the expression vector in order to perform a Gibson assembly, and an *Eco*RI restriction site.

Overexpression of carotenogenic genes

Plasmids harboring a carotenogenic gene (general abbreviation *crt*), pEKEx3-*crt* or pVWEx1-*crt*, allowed an IPTG-inducible overexpression of *crt*. They were constructed on the basis of pEKEx3 (Stansen et al. 2005) or pVWEx1 (Peters-Wendisch et al. 2001), respectively. Amplification of *crt* by PCR from genomic DNA of *C. glutamicum* WT, which was prepared as described (Eikmanns et al. 1995), was carried out using the respective primers (Table S1). The amplification of the *crt* genes from *M. luteus* was based on the plasmid pCRT-E2YgYh-O7 as template. The amplified products were cloned into the appropriately restricted pEKEx3 or pVWEx1 plasmid DNA. The codon-optimized *lbtA* and *lbtBC* genes (KF693237) could be directly transferred from the plasmid provided by Life Technologies by restriction and re-ligation with the desired pEKEx3 plasmid.

Deletion of carotenogenic genes in *C. glutamicum* WT

For deletion of the carotenogenic gene *crt*, the suicide vector pK19*mobsacB* was used (Schäfer et al. 1994). Genomic regions flanking *crt* were amplified from genomic DNA of *C. glutamicum* WT using primer pairs *crt*-A/*crt*-B and *crt*-C/*crt*-D (Table S1), respectively. The PCR products were purified and linked by crossover PCR using the primer pair *crt*-A/*crt*-D (Table S1). The either *Sma*I restricted or purified PCR product was cloned into pK19*mobsacB* resulting in the construction of deletion vector pK19*mobsacB*-*crt* (Table S1). Targeted deletion of *crt* via two-step homologous recombination using this vector was carried out as described previously (Eggeling and Bott 2005). For the first recombination event, integration of the vector into one of the *crt* flanking regions was selected via kanamycin resistance. Integration of the deletion vector into the genome results in a sucrose sensitivity because of the *sacB* gene product levansucrase. Selection for clones that have excised the deletion vector in a second recombination event was carried out via sucrose resistance. Deletion of *crt* was verified by PCR analysis of the constructed mutant using primer pair *crt*-E/*crt*-F (Table S1).

Extraction of carotenoids from bacterial cell cultures

To extract carotenoids from the *C. glutamicum* strains, 15 ml aliquots of the cell cultures were centrifuged at 10,000×*g* for 15 min, and the pellets were washed with deionized H_2O. The pigments were extracted with 10 ml methanol/acetone mixture (7:3) at 60 °C for 30 min with thorough vortexing every 10 min. When necessary, several extraction cycles were performed to remove all visible colors from the cell pellet (Heider et al. 2012).

Analysis of carotenoids

The extraction mixture was centrifuged 10,000×*g* for 15 min and the supernatant was transferred to a new tube. The carotenoid content in the extracts was quantified through absorbance at 470 nm by high-performance liquid chromatography (HPLC) analysis (see below) and the concentrations were calculated using a standard curve and appropriate dilutions. HPLC analyses of the *C. glutamicum* extracts were performed on an Agilent 1200 series HPLC system (Agilent Technologies Sales & Services GmbH & Co. KG, Waldbronn), including a diode array detector for UV/visible (vis) spectrum recording. For separation, a column system consisting of a precolumn (10×4 mm MultoHigh 100 RP18-5, CS Chromatographie Service GmbH, Langerwehe, Germany) and a main column (ProntoSIL 200–5 C30, 250×4 mm, CS Chromatographie Service GmbH, Langerwehe, Germany) was used. Quantification of carotenoids was performed using the extracted wavelength chromatogram at 470 nm for decaprenoxanthin and carotenoids with corresponding UV/vis profiles as well as for lycopene and corresponding carotenoids. Lycopene from tomato (Sigma, Steinheim, Germany) and β-carotene (Merck, Darmstadt, Germany) were used as standards. The carotenoids were dissolved in chloroform according to its solubility and diluted in methanol/acetone (7:3). Due to the lack of appropriate

standards for the C_{50} carotenoids decaprenoxanthin, C.p.450, and sarcinaxanthin, their quantification was calculated based on a β-carotene standard and reported as β-carotene equivalents. The HPLC protocol comprised a gradient elution for 10 min and a mobile phase composition of (A) methanol and (B) methanol/methyl tert-butyl ether/ethyl acetate (5:4:1) starting from 10 to 100 % eluent B followed by 20 min of isocratic elution with 100 % B. After that, the eluent composition is set back to 10 % B for 3 min. The injection volume was 50 μl and the flow rate was kept constant at 1.4 ml/min.

Computational analysis

To compare the cg0730/*crtX* gene product from *C. glutamicum* ATCC 13032 with carotenoid glycosyltransferases from other organisms, the amino acid sequences of the respective genes were obtained from NCBI database (http://www.ncbi.nlm.nih.gov/protein). Sequence comparisons were carried out using BLASTP (www.ncbi.nlm.nih.gov/blast/Blast.cgi,) (Altschul et al. 1990) and Clustal Omega (Goujon et al. 2010). Species names, gene identifier, and accession numbers are given in Table S2. A phylogenetic tree was constructed using the neighbor joining method provided by MEGA version 5 (Tamura et al. 2011) with 1,000 bootstrap replicates.

Results

Overexpression of biosynthetic pathway genes resulted in up to 20-fold increased production of the native C_{50} carotenoid decaprenoxanthin in *C. glutamicum*

C. glutamicum WT produces only little decaprenoxanthin and it was of interest to test if the production level can be increased by rational genetic engineering. The *crtE* gene encoding the enzyme catalyzing the initial step of the carotenoid synthesis pathway, synthesis of GGPP (see Fig. 1), was overexpressed in *C. glutamicum* WT. This entailed an about twofold higher decaprenoxanthin accumulation than that observed for the empty vector control strain (Fig. 2). To further enhance carotenogenesis, all three lycopene biosynthesis genes, *crtE*, *crtB*, and *crtI* (Fig. 2), were co-expressed as a synthetic operon in the IPTG-inducible expression vector pVWEx1. Since *crtB* and *crtI* are overlapping in their sequences, they have been cloned as they are clustered in the genome. The chosen gene order followed the sequence of the encoded enzymes in the pathway and artificial ribosome binding sites were introduced before *crtE* and *crtBI* (see "Material and methods" section). Decaprenoxanthin formation by *C. glutamicum* WT(pVWEx1-*crtEBI*)(pEKEx3) was comparable to that of the empty vector control; however, the former strain also accumulated certain pathway intermediates, in particular lycopene (Fig. 2), which resulted in an orange to red appearance of the cells. Thus, since elongation of lycopene appeared to be a bottle neck in the biosynthetic pathway for decaprenoxanthin, a second compatible plasmid, pEKEx3-*crtYEb*, was constructed and transformed to *C. glutamicum* WT. The overexpression of the genes *crtY* and *crtEb*, encoding the carotenoid ε-cyclase and the lycopene elongase, respectively, enhanced the conversion of lycopene to decaprenoxanthin. Analysis of *C. glutamicum* WT(pVWEx1-*crtEBI*)(pEKEx3-*crtYEb*) showed that the decaprenoxanthin production level was 20-fold increased as compared to the empty vector control (Fig. 2). We noticed that this strain still accumulated some lycopene, suggesting a potential for even higher decaprenoxanthin production yields in this host.

Construction of the lycopene overproducing platform strain *C. glutamicum* LYC1

Most natural carotenoids including decaprenoxanthin are derived from lycopene (Fig. 1). It was therefore desired to establish a genetically defined lycopene overproducing platform *C. glutamicum* strain with application as a valuable host for further genetic engineering aiming at producing alternative carotenoids. Previously, we have demonstrated that deletion of the lycopene elongase gene *crtEb* to avoid conversion of lycopene to flavuxanthin, coupled to overexpression of the lycopene biosynthesis genes *crtE*, *crtB*, and *crtI*, entailed lycopene accumulation to 2.4±0.3 mg/g CDW in *C. glutamicum* (Heider et al. 2012). To construct *C. glutamicum* LYC1, the lycopene elongase encoding gene *crtEb* and the *crtY* genes for conversion of flavuxanthin to decaprenoxanthin were deleted and *crtE*, *crtB*, and *crtI* were expressed as synthetic operon from plasmid pVWEx1-*crtEBI*. The resulting strain *C. glutamicum* Δ*crtYEb*(pVWEx1-*crtEBI*) was named LYC1 and shown to produce lycopene up to 0.79±0.09 mg/g CDW in minimal medium with 100 mM glucose, which is about 11 times more than in the Δ*crtYEb* deletion strain and more than 120 times more than *C. glutamicum* WT. LYC1 should therefore represent a lycopene overproducing platform strain for production of various lycopene-derived carotenoids simply by introducing a compatible plasmid comprising the relevant genes for lycopene conversion (see below).

LYC1-based production of the C_{50} carotenoids decaprenoxanthin, C.p.450, and sarcinaxanthin

LYC1-based production of decaprenoxanthin was tested by transformation of LYC1 with pEKEx3-*crtYEb* to yield *C. glutamicum* strain DEC1 (see Table 1). Analysis of DEC1 demonstrated that it produced about 2 mg/g CDW of

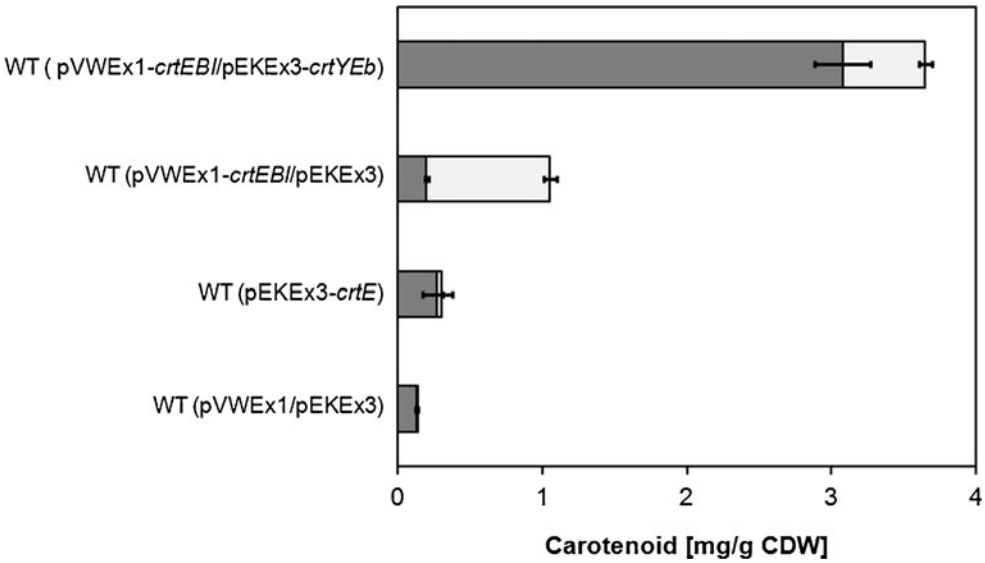

Fig. 2 Decaprenoxanthin production by *C. glutamicum* WT transformed with the indicated plasmids. Concentrations of decaprenoxanthin (*dark gray bars*) and residual lycopene are depicted (*light gray bars*). Mean values and standard deviations of three replicate cultivations in glucose minimal medium with 1 mM IPTG are given. Decaprenoxanthin quantities are given as β-carotene equivalents

decaprenoxanthin (Fig. 3). We noticed that the cells were orange indicating significant accumulation of lycopene. In an attempt to provide higher lycopene elongase levels, the order of genes *crtY* and *crtEb* was inverted and a ribosome binding site was inserted in front of each gene. Transformation of *C. glutamicum* LYC1 with the resulting plasmid pEKEx3-*crtEbY* yielded strain DEC2. Cells of *C. glutamicum* DEC2 showed a more intense yellow color than WT (Fig. S1) and overproduced decaprenoxanthin (Fig. 3), while lycopene could not be detected. However, the growth rate of DEC2 was only 0.01 h^{-1} under the chosen conditions (Fig. 3). To alleviate growth perturbation of DEC2, cultivations were induced with only 50 μM IPTG and as a consequence a growth rate of 0.18 h^{-1} and almost 30-fold elevated decaprenoxanthin production as compared to the WT resulted (Fig. 3). Decaprenoxanthin production by DEC1 was also increased under these conditions (Fig. 3). However, pathway intermediates like lycopene were still accumulating. When induced with 50 μM IPTG, *C. glutamicum* DEC1 accumulated decaprenoxanthin to concentrations of 3.5±0.2 mg/g CDW and *C. glutamicum* DEC2 to 3.9±0.2 mg/g (Fig. 3).

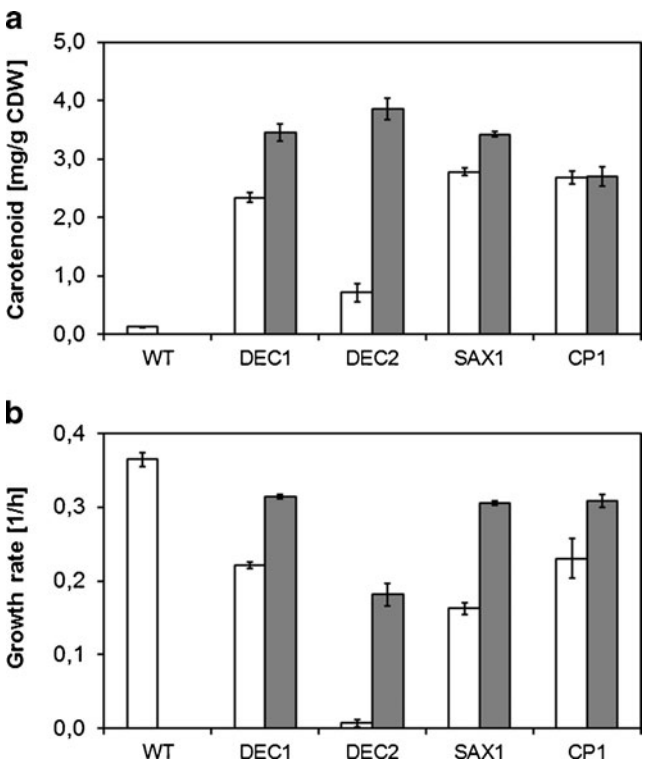

Fig. 3 Production of the C_{50} carotenoids decaprenoxanthin, sarcinaxanthin, and C.p.450 by *C. glutamicum* WT and recombinant *C. glutamicum* LYC1-based strains. **a** Accumulation of decaprenoxanthin by strains WT, DEC1, and DEC2; of sarcinaxanthin by SAX1; and of C.p. 450 by CP1. C_{50} carotenoid quantities are given as β-carotene equivalents. **b** Growth rates. Cells were grown in glucose CGXII minimal medium for 24 h induced by 1 mM (*white bars*) or 50 μM (*gray bars*) IPTG. Mean values and standard deviations of three replicates are given

Next, it was of interest to test if our platform strain can also be used to produce the heterologous C_{50} carotenoids sarcinaxanthin and C.p.450. LYC1 was therefore transformed with plasmids encoding the respective lycopene-converting enzymes from *M. luteus* and *Dietzia* sp. CQ4. The genes *crtE2* and $crtY_{g/h}$, the latter herein referred to as $crtY_{Ml}$, from *M. luteus*, have been amplified from pCRT-E2YgYh-O7 (Netzer et al. 2010) and introduced into pEKEx3 as a gene cluster with an artificial ribosome binding site in front of *crtE2* to yield pEKEx3-*crtE2Y*. The genes *lbtA* and *lbtBC* from *Dietzia* sp. CQ4 have been codon optimized for *C. glutamicum*, synthesized, and cloned into pEKEx3 with a ribosome binding site in front of each gene. Transformation of *C. glutamicum* LYC1 with pEKEx3-*crtE2Y* and pEKEx3-*lbtABC* yielded strains SAX1 and CP1, respectively. *C. glutamicum* SAX1 accumulated high levels of sarcinaxanthin

(Fig. 3). Since the γ-cyclic sarcinaxanthin absorbs light at wavelengths of about 415, 440, and 470 nm as the ε-cyclic decaprenoxanthin (Table S3), cells of *C. glutamicum* SAX1 showed a bright yellow color (Fig. S1). *C. glutamicum* CP1 cells produced C.p.450 (Fig. 3) and appeared slightly orange (Fig. S1) as the β-cyclic C.p.450 absorbed light at higher wavelengths (Table S3) due to its higher extent of π-electron delocalization along the carbon backbone. Notably, neither SAX1 nor CP1 accumulated lycopene indicating complete conversion of this precursor to the respective heterologous C_{50} carotenoids (Fig. 3). Reducing the IPTG concentration from 1 mM to 50 μM improved growth rates of both strains and led to about 25 % increased accumulation of sarcinaxanthin by *C. glutamicum* SAX1 (Fig. 3). With 50 μM IPTG, *C. glutamicum* CP1 produced 2.7 mg/g± 0.2 mg/g CDW C.p.450 and *C. glutamicum* SAX1 produced 3.4±0.1 mg/g CDW sarcinaxanthin; both are in the same range as the decaprenoxanthin production levels obtained under these conditions (see above). These results strongly indicate that balanced and fine-tuned gene expression is crucial for optimized carotenoid production. Thus, *C. glutamicum* LYC1 appears to be a suitable platform strain for the production of endogenous and heterologous C_{50} carotenoids.

Putative glucosyl transferase in *C. glutamicum* encoded by cg0730

C. glutamicum can synthesize both mono- and diglucosylated decaprenoxanthin, but the respective glucosyl transferase has not yet been characterized (Krubasik et al. 2001b). Of the few biochemically characterized carotenoid glycosyl transferases, GT-A fold glycosyl transferases, e.g., from *Staphylococcus aureus* or *Chlorobium tepidum*, are known to differ with respect to their acceptor molecules (C40/C30 carotenoids such as diaponeurosporenoate or chlorobactene) and the transferred sugar (rhamnose, glucose, fucose) (Pelz et al. 2005; Maresca and Bryant 2006). On the other hand, specific GT-B fold zeaxanthin glucosyltransferases can be found, e.g., in *P. ananatis* (Coutinho et al. 2003; Yokoyama et al. 1998). CrtX of *M. luteus* was shown to glucosylate the C_{50} carotenoid sarcinaxanthin and is encoded in its *crt* cluster (Netzer et al. 2010). A similar gene is part of the *crt* cluster in *Dietzia*, whereas in *C. glutamicum* a putative *crtX* homolog (cg0730) is found about 4.5 kb upstream of its *crt* cluster.

A phylogenetic comparison of Cg0730/CrtX from *C. glutamicum* with biochemically characterized carotenoid glycosyl transferases revealed two clusters (Fig. 4). Cluster 1 comprised all GT-A fold glycosyl transferases with a subcluster of the CrtX proteins from *C. glutamicum*, *Dietzia*, and *M. luteus* and a distinct subcluster with the C_{30}/C_{40} carotenoid glycosyl transferases from *S. aureus*, *Chlorobium tepidum*, *Synechococcus* sp., and *Gemmatimonas aurantiaca* (Graham and Bryant 2009; Maresca and Bryant 2006; Pelz et al. 2005; Takaichi et al. 2010). Cluster 2 comprised all GT-B fold glycosyl transferases (Hundle et al. 1994; Misawa et al. 1990; Sedkova et al. 2005; Seo et al. 2009) and MgtA from *C. glutamicum*. Clustering of the putative CrtX protein from *C. glutamicum* with glycosyl transferases from other C_{50} carotenoid containing bacteria, but distinct from the C_{30}/C_{40} carotenoid glycosyl transferases (Fig. 4), supported the hypothesis that it might be involved in glucosylation of the endogenous C_{50} carotenoid decaprenoxanthin.

Glucosylation of C_{50} carotenoids in *C. glutamicum*

Deletion of *crtX* in *C. glutamicum* WT and in the carotenoid overproducer strains LYC1, DEC2, SAX1, and CP1 yielded strains Δ*crtX*, LYC2, DEC3, SAX2, and CP2, respectively (see Table 1). All these strains devoid of *crtX* grew similar as the parental strains in LB or minimal medium with glucose. The absence of any carotenoid glucosylation was verified by reversed phase (RP)-HPLC (Fig 5). RP-HPLC analysis of cell extracts from strain Δ*crtX* revealed a peak in the chromatogram with longer retention times (rt) with respect to the glucosylated form as expected (Fig. 5a, Table S3). Plasmid-borne expression of endogenous *crtX* complemented this phenotype as glucosylated decaprenoxanthin accumulated (Fig. 5a). Also when the gene coding for the sarcinaxanthin glucosyltransferase $CrtX_{Ml}$ from *M. luteus* (Netzer et al. 2010) was expressed in strain Δ*crtX*, accumulation of glucosylated decaprenoxanthin was re-established (Fig. 5a). Interestingly, heterologous expression of the gene for the GT-B fold zeaxanthin glucosyl transferase from *P. ananatis* (Misawa et al. 1990) resulted in production of glucosylated decaprenoxanthin (Fig. 5a).

C. glutamicum strain Δ*crtX*Δ*crtYEb* expressing either *crtE2Y* from *M. luteus* or *lbtABC* from *Dietzia* sp. produced non-glucosylated sarcinaxanthin and C.p.450, respectively (Fig. 5b). When *crtX* from *C. glutamicum* was expressed from a plasmid in these strains, production of glucosylated sarcinaxanthin and C.p.450, respectively, was obtained (Fig. 5b). Thus, CrtX from *C. glutamicum* is an active decaprenoxanthin, sarcinaxanthin, and C.p.450 glucosyl transferase.

Production and glucosylation of C_{40} carotenoids by recombinant *C. glutamicum*

After having demonstrated that *C. glutamicum* strain LYC1 and its *crtX* deletion derivative LYC2 can be applied for the production of lycopene-derived C_{50} carotenoids (see above), it was attempted to produce C_{40} carotenoids. Therefore, $crtY_{Pa}$ (PANA_4160) encoding lycopene cyclase which catalyses conversion of lycopene to β-carotene from *P. ananatis* was expressed alone or with $crtZ_{Pa}$ (PANA_4163) which encodes

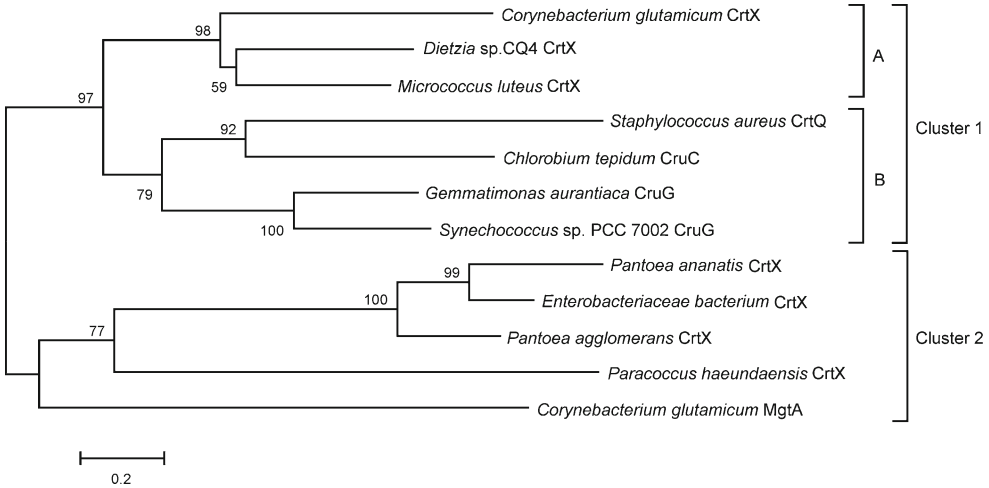

Fig. 4 Phylogenetic relation of carotenoid glycosyltransferases. The sequence alignment was carried out using Clustal Omega, the phylogenetic tree analysis was performed with MEGA version5 and the phylogenetic tree was constructed using the neighbor-joining method. The functionally unrelated GDP-mannose-dependent 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

β-carotene hydroxylase in LYC2 to yield strains BCR and ZEA. Cells of both strains, *C. glutamicum* BCR and ZEA, were orange (Fig. S1) and produced carotenoids with absorption maxima between 420 and 480 nm (Table S3). *C. glutamicum* BCR accumulated 4.0±0.5 mg/g CDW β-carotene, while *C. glutamicum* ZEA produced a mixture of β-carotene (3.0 mg/g±0.5 mg/g CDW) and non-glucosylated zeaxanthin (0.9±0.1 mg/g CDW) (Table 2). These results demonstrated that *C. glutamicum* indeed can produce C_{40} carotenoids and at the same levels as for the C_{50} carotenoids (see above).

In an attempt to produce the C_{40} carotenoid zeaxanthin in its glucosylated form by *C. glutamicum*, *crtX* genes from *C. glutamicum*, *M. luteus*, and *P. ananatis* were expressed in the zeaxanthin-producing strain Δ*crtX*Δ*crtYEb*. Interestingly, expression of *crtX* from *P. ananatis* allowed for production of glucosylated zeaxanthin (Fig. 5c), while glucosylated zeaxanthin was not observed upon expression of *crtX* genes from the former two organisms. Taken together, these results show that it is possible to produce β-carotene and either glucoslyated or non-glucosylated zeaxanthin by strains based on the *C. glutamicum* platform strain LYC1.

Discussion

In this paper, we demonstrate that *C. glutamicum* is a high potential platform organism for carotenoid production. The C_{50} carotenoids decaprenoxanthin, C.p.450, and sarcinaxanthin as well as the C_{40} carotenoids β-carotene and zeaxanthin could be overproduced with considerable yields either in their glucosylated or non-glucosylated forms. *C. glutamicum* is advantageous as production host since it is a workhorse in biotechnology, is genetically amenable, and has a history of more than 50 years of safe use in the large-scale food and feed production. The already broad spectrum of carbon sources for growth and production has recently been widened for access to glycerol from the biodiesel process (Meiswinkel et al. 2013), to pentoses (Gopinath et al. 2011), to amino sugars (Uhde et al. 2013), to starch (Seibold et al. 2006; Song et al. 2013), and β-glucans (Tsuchidate et al. 2011), thus, offering several upstream options for large-scale production.

Previously, we have shown that engineering of the terminal carotenoid pathway of *C. glutamicum* resulted already in considerable yields of lycopene (Heider et al. 2012). Production in the milligram per gram CDW range of lycopene, β-carotene, zeaxanthin, C.p.450, decaprenoxanthin, and sarcinaxanthin by recombinant *C. glutamicum* strains described here compares favorably with other production hosts where only the terminal pathway had been optimized. For example, sarcinaxanthin production (~3.5 mg/g CDW) by *C. glutamicum* SAX1 was higher than reported for *E. coli* (2.5 mg/g CDW; (Netzer et al. 2010)). Moreover, *C. glutamicum* strain BCR produced ~4 mg/g CDW β-carotene which is superior to production of ~1.6 mg/g CDW β-carotene by a *Saccharomyces cerevisiae* strain with engineered terminal carotenoid pathway and comparable to ~5.9 mg/g CDW by a derived yeast strain with increased precursor supply (Verwaal et al. 2007). Similarly, production of β-carotene by recombinant *E. coli* of ~1.5 mg/g CDW was improved ~3.5 fold by increasing supply of IPP in the MEP pathway (Zhao et al. 2013). Modulating expression of genes in the TCA cycle, ATP synthesis, and the pentose phosphate shunt led to a 65 % increase compared to the parental *E. coli* strain (Zhao et al. 2013). Thus, the already high carotenoid

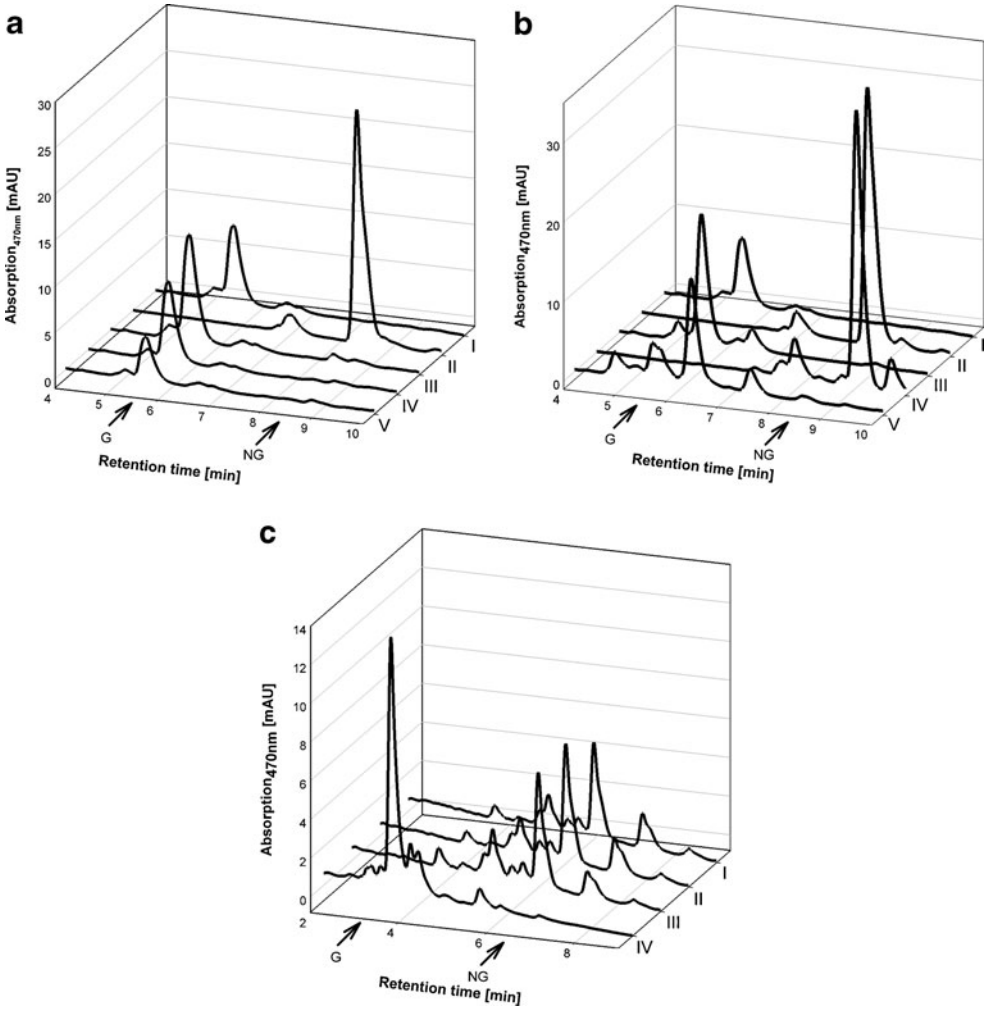

Fig. 5 Glucosylation of decaprenoxanthin, sarcinaxanthin, C.p.450, and zeaxanthin by CrtX from *C. glutamicum*, *M. luteus*, or *P. ananatis*. Glucosylation of decaprenoxanthin (**a**). HPLC traces of extracts from *C. glutamicum* strains WT(pVWEx1)(pEKEx3) (*I*), Δ*crtX*(pEKEx3) (*II*), Δ*crtX*(pEKEx3-*crtX*$_{Cg}$) (*III*), Δ*crtX*(pEKEx3-*crtX*$_{Ml}$) (*IV*), and Δ*crtX*(pEKEx3-*crtX*$_{Pa}$) (*V*) are shown. Glucosylation of sarcinaxanthin and C.p. 450 (**b**). HPLC traces of extracts from *C. glutamicum* strains WT(pVWEx1)(pEKEx3) (*I*), SAX2(pVWEx1) (*II*), SAX2(pVWEx1-*crtX*$_{Cg}$) (*III*), CP2(pVWEx1) (*IV*), and CP2(pVWEx1-*crtX*$_{Cg}$) (*V*). Glucosylation of zeaxanthin (**c**). HPLC traces of extracts from *C. glutamicum* strains ZEA(pVWEx1) (*I*), ZEA(pVWEx1-*crtX*$_{Cg}$) (*II*), ZEA(pVWEx1-*crtX*$_{Ml}$) (*III*), and ZEA(pVWEx1-*crtX*$_{Pa}$) (*IV*). For details of HPLC analysis, see "Material and methods"

production by the constructed *C. glutamicum* strains as consequence of terminal pathway engineering may be further increased by optimizing precursor supply and modulating central metabolism.

Table 2 Production of the C_{40} carotenoids β-carotene and zeaxanthin by various *C. glutamicum* strains

	Carotenoid concentration [mg/g CDW]	
Strain	β-Carotene	Zeaxanthin
WT(pVWEx1/pEKEx3)	<0.01	<0.01
Δ*crtX*Δ*crtYEb* (pVWEx1/pEKEx3-*crtY*$_{Pa}$)	0.09±0.01	n.d.
Δ*crtX*Δ*crtYEb* (pVWEx1-*crtEBI*/pEKEx3-*crtY*$_{Pa}$)=BCR	3.95±0.52	n.d.
Δ*crtX*Δ*crtYEb* (pVWEx1/pEKEx3-*crtYZ*$_{Pa}$)	n.d.	0.04±0.00
Δ*crtX*Δ*crtYEb* (pVWEx1-*crtEBI*/pEKEx3-*crtYZ*$_{Pa}$)=ZEA	3.15±0.09	0.88±0.05

Cells were grown in glucose CGXII minimal medium for 24 h induced by 1 mM IPTG. Means and standard deviations of three replicates are reported.
n.d. not determined

The highest lycopene accumulation of 2.4 mg/g observed in *C. glutamicum* (Heider et al. 2012) is lower than the highest accumulation of up to 4 mg/g of, e.g., sarcinaxanthin, decaprenoxanthine, or β-carotene. While linear lycopene incorporates into the cytoplasma membrane in a disordered manner, longer carotenoids, especially those with polar terminal groups, are supposed to span through the membrane leading to a more dense packed organization (Britton 2008).

We have shown that accumulation of C_{50} carotenoid pathway intermediates could be avoided by overexpression of the *crtY* and *crtEb* genes in a synthetic operon in the same sequential order as the encoded enzymes function in the decaprenoxanthin pathway, which may reflect the principle of a "just-in-time transcription program in metabolic pathways" observed for induction of amino acid biosynthesis pathways in *E. coli* (Zaslaver et al. 2004). Interestingly, using a similar strategy for efficient conversion of β-carotene to zeaxanthin by overexpressing *crtY* and *crtZ* from *P. ananatis*, in the same sequential order as the encoded enzymes function in zeaxanthin biosynthesis, did not result in increased zeaxanthin accumulation (see Table 2). β-Carotene hydroxylase (CrtZ) has also been identified as rate-limiting step in astaxanthin production (Choi et al. 2006). Since efficient conversion of β-carotene to zeaxanthin and to astaxanthin was observed employing CrtZ from *Brevundimonas* sp. SD212 (Choi et al. 2006), expression of *crtZ* from a different source might improve zeaxanthin production by *C. glutamicum* strain ZEA. Besides balancing the ratio of *crtY* and *crtEb* overexpression (see above), lower absolute overexpression by reducing the inducer concentration proved beneficial since this enabled fast growth and, thus, high productivities. Similar strategies have been applied in *E. coli* (Lale et al. 2011; Zhao et al. 2013).

As shown here, CrtX from *C. glutamicum* and from *M. luteus* glucosylate decaprenoxanthin, C.p.450, and sarcinaxanthin, but not zeaxanthin. Phylogenetic analysis indicated that both CrtX proteins belong to GT-A fold glucosyl transferases, which differ in their primary structure from the longer GT-B fold glucosyl transferases such as zeaxanthin glucosyl transferase of *P. ananatis* with which they share less than 20 % identical amino acids (Coutinho et al. 2003) and also differ in substrate specificity. While it was known that CrtX from *P. ananatis* glucosylates the β-rings of zeaxanthin and astaxanthin in *para*-position (Misawa et al. 1990; Yokoyama et al. 1998), we found that this enzyme also glucosylates the hydroxymethyl group of the terminal isoprene unit of decaprenoxanthin and this was first reported here. Thus, CrtX from *P. ananatis* glucosylates C_{40} and C_{50} carotenoids with hydroxyl groups at aliphatic and ring positions.

Since upon deletion of *crtX* no phenotypical changes were observed, the physiological role of the decaprenoxanthin glucosylation in *C. glutamicum* remains unknown. Glucosylation of zeaxanthin is thought to improve membrane integration as it spans across the membrane and its glucosylated termini interact with the polar lipid head groups of the cell membranes (Britton 2008). The water solubility of diglucosylated zeaxanthin of 800 ppm is much higher than that of non-glucosylated zeaxanthin at 12.6 ppm (Hundle et al. 1992) and it is likely that glucosylation of other carotenoids affects their water solubility similarly. Thus, production of glucosylated carotenoid derivatives might be beneficial for industrial purposes. Since *C. glutamicum* strains for production of non-glucosylated and glucosylated decaprenoxanthin, C.p.450, sarcinaxanthin, and zeaxanthin are now available, simplification of down-stream processing and carotenoid purification might be important steps to further increase productivity.

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