Optimization of the IPP precursor supply for the production of lycopene, decaprenoxanthin and astaxanthin by Corynebacterium glutamicum

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The biotechnologically relevant bacterium Corynebacterium glutamicum, currently used for the million ton-scale production of amino acids for the food and feed industries, is pigmented due to synthesis of the rare cyclic C50 carotenoid decaprenoxanthin and its glucosides. The precursors of carotenoid biosynthesis, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate, are synthesized in this organism via the methylethylthritol phosphate (MEP) or non-mevalonate pathway. Terminal pathway engineering in recombinant C. glutamicum permitted the production of various non-native C50 and C40 carotenoids. Here, the role of engineering isoprenoid precursor supply for lycopene production by C. glutamicum was characterized. Overexpression of dks encoding the enzyme that catalyzes the first committed step of the MEP-pathway by chromosomal promoter exchange in a prophage-cured, genome-reduced C. glutamicum strain improved lycopene formation. Similarly, an increased IPP supply was achieved by chromosomal integration of two artificial operons comprising MEP pathway genes under the control of a constitutive promoter. Combined overexpression of dks and the other six MEP pathways genes in C. glutamicum strain LYC3-MEP was not synergistic with respect to improving lycopene accumulation. Based on C. glutamicum strain LYC3-MEP, astaxanthin could be produced in the milligrams per gram dry cell weight range when the endogenous genes crtE, crtB, and ctfI for conversion of geranylgeranyl pyrophosphate to lycopene were coexpressed with the genes for lycopene cyclase and β-carotene hydroxylase from Pantoea ananatis and carotene C(4) oxygenase from Brevundimonas aurantiaca.

Keywords: carotenoid production, genome-reduced Corynebacterium glutamicum, MEP pathway, synthetic operons, astaxanthin

INTRODUCTION

Carotenoids are ubiquitous natural pigments with colors ranging from yellow to red. They are composed of isoprene units and belong to the family of terpenoids. These pigments do not only play important and versatile roles in their biological hosts, but are also suggested to have a beneficial effect on human health. Furthermore, they are intensively applied for food and beverage coloration (Downham and Collins, 2000; Gassel et al., 2013). Hence, carotenoids have received extensive considerable attention and especially the interest for an efficient and environmental-friendly production by microbial hosts is increasing (Lee and Schmidt-Dannert, 2002; Das et al., 2007; Harada and Misawa, 2009; Cutzu et al., 2013). In order to compete with already existing production processes, such as chemical synthesis or extraction from organic material, the large-scale production in microbial hosts requires process as well as strain optimization. One of the most common strategies for enhanced production is the efficient supply of precursor molecules as all carotenoids derive from the universal C5 precursor molecule IPP and its isomer DMAPP. IPP and DMAPP can be synthesized via two independent pathways, the mevalonate (MVA) and the 2-methylerythritol 4-phosphate (MEP) pathway (Rodriguez-Concepcion and Boronat, 2002). The MVA pathway starts from acetyl-CoA and operates mainly in eukaryotes (mammals, fungi, in the cytoplasm of plant cells), archaea, and a limited number of bacteria. The MEP pathway that starts from pyruvate and glyceraldehyde 3-phosphate and proceeds via the eponymous intermediate MEP was identified much later (Rohmer et al., 1993) and is found in most bacteria as well as in plant plastids (Rohmer, 1999; Lange et al., 2000; Lee and Schmidt-Dannert, 2002). Both pathways also differ regarding redox and energy requirements (Steinbüchel, 2003). As the MEP pathway is present in several pathogens such as Plasmodium falciparum and Mycobacterium tuberculosis, but not in mammals, it is considered a drug target (Jomaa et al., 1999; Tester and Brown, 2003).

The MEP pathway consists of nine reactions catalyzed by eight enzymes (Figure 1) starting with the transfer of an acetaldehyde group derived from pyruvate to GAP, forming 1-deoxy-d-xylulose 5-phosphate (DXP), in the reaction of DXP synthase Dxs (EC 2.2.1.7). The intermediate DXP is also the precursor for thiamine (vitamine B1) (Begley et al., 1999) and pyridoxal (vitamine B6) (Hill et al., 1996) biosynthesis. Subsequently, DXP reductoisomerase Dxr (EC 1.1.1.267) converts DXP to MEP using NADPH as cofactor. MEP is then converted to the cyclic diphosphate 2C-methyl-d-erythritol-2,4-cyclophosphosphate (ME-CP) by the three
enolizes IspD, IspE, and IspF (Gräwert et al., 2011). ME-cPP is then converted to IPP and DMAPP by a reduction and elimination reaction catalyzed by the two iron–sulfur proteins IspG and IspH (Rohdich et al., 2004). It is proposed that flavodoxin is an essential redox partner for one of the enzymes (Adam et al., 2002; Gräwert et al., 2004; Puan et al., 2005). IPP and DMAPP can be synthesized independently by IspH (Gräwert et al., 2004). IPP and DMAPP often do not occur in the same ratio as for example in Escherichia coli IPP is synthesized in a 5:1 proportion to DMAPP (Rohdich et al., 2002; Gräwert et al., 2004; Xiao et al., 2008). The IPP:DMAPP isomerase Idi (EC 5.3.3.2) facilitates the isomerization between IPP and DMAPP. In the case of microorganisms using the MVA pathway produce/synthesize IPP exclusively, isomerases are essential enzymes, whereas in bacteria possessing the MEP pathway idi is not essential for the survival of the cells (Hahn et al., 1999; Jüssing et al., 2007).

Corynebacterium glutamicum is a pigmented Gram-positive bacterium with a long and safe history in the food and feed sector as it is used for the fermentative production of amino acids. Annually, about 2.6 million tons of l-glutamate and about 1.95 million tons of l-lysine are produced biotechnologically worldwide (Ajinomoto, Food Products Business. Available from http://www.ajinomoto.com/en/ir/pdf/Food-Oct2012.pdf and /Feed-useAA-Oct2013.pdf, Cited 18 March 2014). Besides amino acids, the diamines cadaverine and putrescine (Mimitsuka et al., 2007; Schneider and Wendisch, 2010) and the alcohols ethanol and isobutanol (Sakai et al., 2007; Blombach and Eikmanns, 2011), among others, can be produced from sugars by recombinant C. glutamicum strains. Furthermore, access of C. glutamicum to alternative feed stocks like glycerol from the biodiesel process (Meiswinkel et al., 2013), pentoses from lignocellulosics (Gopinath et al., 2011), amino sugars (Uhde et al., 2013; Matano et al., 2014), starch (Seibold et al., 2006), and β-glucans (Tsuchidate et al., 2011) has been engineered.

Recently, the potential of C. glutamicum for production of carotenoids has been explored. C. glutamicum synthesizes the cyclic C50 carotenoid decaprenoxanthin and its glucosides (Figure 1). Its carotenogenic pathway and the respective genes have been elucidated (Krubasik et al., 2001; Heider et al., 2012, 2014a) and overproduction of the C50 carotenoids decaprenoxanthin, sarcinaxanthin, and C.p. 450 in the milligrams per gram cell dry weight (DCW) range by C. glutamicum was achieved by metabolic engineering of the terminal carotenoid pathway (Heider et al., 2014a). Moreover, the heterologous production of the C40
carotenoids β-carotene and zeaxanthin could be established (Heider et al., 2014a) and hydroxylated carotenoids could be produced either as aglycons or as di-glucosides (Heider et al., 2014a). Engineering of C. glutamicum for the production of a sesquiterpene, (+)-valencene, was possible as well (Frohwittet al., 2014).

Based on its genome sequence, all genes of the MEP pathway of C. glutamicum have been putatively assigned. However, neither have the respective genes or enzymes of the MEP pathway been functionally analyzed nor has engineering for an increased IPP supply been reported. The MEP pathway genes are distributed over the genome of C. glutamicum. The MEP pathway genes dxs (cg2083), ispH (cg1164), and idi (cg2531) are monocistronic, while dtxr (cg2208), ispD (cg2945), ispE (cg1039), ispF (cg2944), and ispG (cg2206) belong to operons. IspE is the third gene of the operon cg1037-ksgA-ispE-cg1040-pdxK with genes for a putative resuscitation-promoting factor (cg1037), putative dimethyladeno- sine transferase KsgA, and putative pyridoxamine kinase PdxK. IspD and ispF are encoded in the cg2946-ispDF operon with cg2946, which codes for a CarD-like transcriptional regulator. Dtxr and ispG are organized in a transcriptional unit separated by an uncharacterized gene (cg2207) putatively encoding a membrane-embedded Zn-dependent protease. In bacteria, two bottlenecks in the MEP pathway were proposed. On the one hand, DXP synthase, which catalyzes the first reaction is claimed to be rate-limiting (Sprenger et al., 1997; Xiang et al., 2007) and is essential in E. coli (Sauret-Gueto et al., 2003) and Bacillus subtilis (Julsing et al., 2007) and possibly further bacteria. On the other hand, overproduction of idi, which is not essential in bacteria possessing the MEP pathway (Hahn et al., 1999; Julsing et al., 2007), improved carotenoid production (Harker and Bramley, 1999; Kim and Keasling, 2001).

In this study, two synthetic operons (ispDFE and dtxr-ispGH) under control of the strong promoter P_{nag} of the C. glutamicum translation elongation factor EF-Tu gene were integrated into the prophase-cured, genome-reduced C. glutamicum strain MB001 (Baumgart et al., 2013). Furthermore, dxs was overexpressed from the chromosome by exchanging the endogenous promoter with the P_{nag} promoter. Finally, idi was overexpressed from an IPTG-inducible plasmid. The genome-reduced strain overexpressing all of the eight MEP pathway genes was then shown to be suitable for production of lycopene and endogenous decaprenoxanthin as well as for production of the non-native astaxanthin.

### MATERIALS 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), for metabolic engineering the prophase-cured C. glutamicum MB001 (Baumgart et al., 2013) was used as platform strain. Precultivation of C. glutamicum strains was performed in LB medium or LB with glucose. For cultivation in CGXII medium (Ebbing 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). Alternatively, cultivations were performed in 1 ml volume in microtiterplates at 1100 rpm at 30°C using Biolector® micro fermentation system (m2p-labs GmbH, Baesweiler, Germany). For cloning, E. 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⁻¹, 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 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 Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table 2. 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.

#### DELETION OF CAROTENOGENIC GENES IN C. GLUTAMICUM MB001

For deletion of the carotenogenic genes crtY_{e/f} and crtEb, encoding the C45/C50 carotenoid θ-cyclase and the lycopene elongase, respectively, the suicide vector pK19 mobscB was used (Schäfer et al., 1994). Genomic regions flanking the crtY_{e/f} cluster were amplified from genomic DNA of C. glutamicum WT using primer pairs crtY_{e/f-crtY}_{e/f} and crtEb-{crtEb}_{e/f} (Table 2), respectively. The PCR products were purified and linked by crossover PCR using the primer pair crtY_{e/f-crtEb}_{e/f} (Table 2). The purified PCR product was cloned into pK19 mobscB resulting in the construction of deletion vector pK19 mobscB-{crtEb}_{e/f} (Table 1). The targeted deletion of crtY_{e/f} via two-step homologous recombination as well as the selection for the first and second recombination events were carried out as described previously (Eggeling and Bott, 2005). Deletion of crtY_{e/f} was verified by PCR analysis of the constructed mutant using primer pair crtY_{e/f-crtEb}_{e/f} (Table 2).
Table 1 | Strains and plasmids used in this study.

| Strain, plasmid | Relevant characteristics | Source or reference |
|-----------------|--------------------------|---------------------|
| **C. glutamicum STRAINS** | | |
| WT | ATCC 13032 | Abe et al. (1967) |
| MB001 | Prophage-cured ATCC 13032; in-frame deletion of prophages cgp1 (cg1507-cg1524), cgp2 (cg1746-cg1752), and cgp3 (cg1890-cg2071) | Baumgart et al. (2013) |
| LYC3 | crtYyEb deletion mutant of *C. glutamicum* MB001 | This work |
| LYC3-Pdxs | LYC3 derivative with dxs (cg2083) under control of the P_tuf promoter integrated into the intergenic region of cg2083 and cg2084 | This work |
| LYC3-Op1 | LYC3 derivative with ispD (cg2945), ispF (cg2944), and ispE (cg1039) under control of the P_tuf promoter integrated into the cg22 cured region between cg1745 and cg1753 | This work |
| LYC3-Op2 | LYC3 derivative with dvr (cg2208), ispG (cg2206), and ispH (cg1164) under control of the P_tuf promoter integrated into the cg1 cured region between cg1506 and cg1525 | This work |
| LYC3-Op1Op2 | LYC3-Op2 derivative with with dxs (cg2083) under control of the P_tuf promoter integrated into the intergenic region of cg2083 and cg2084 | This work |
| LYC3-MEP | LYC3-Op1Op2 derivative with with dxs (cg2083) under control of the P_tuf promoter integrated into the intergenic region of cg2083 and cg2084 | This work |
| **OTHER STRAINS** | | |
| *E. coli* DH5α | F− thi-1 endA1 hsdR17 (r− m−) supE44 ΔlacU169 (φ80lacZΔM15) recA1 gyrA96 relA1 | Hanahan (1983) |
| Pantoea ananatis | ATCC 19321 | Misawa et al. (1990) |
| Brevundimonas aurantiaca | ATCC 15266 | Abraham et al. (1999) |
| **PLASMIDS** | | |
| pK19mobsacB | KmR; *E. coli/C. glutamicum* shuttle vector for construction of insertion and deletion mutants in *C. glutamicum* (pK18 onIVEc sacB lacZα) | Schäfer et al. (1993) |
| pK19mobsacB−ΔcrtYEb | pK19mobsacB with a crtYyEb deletion construct | Heider et al. (2014a) |
| pK19mobsacB-Pdxs | pK19mobsacB derivative with a tuf promoter region (200 bp upstream of the coding sequence of the tuf gene) (cg0587) construct for the promoter exchange of dxs | This work |
| pK19mobsacB-Op1 | pK19mobsacB derivative containing the artificial operon ispDFE under the control of the P_tuf promoter with an additional ribosome binding site in front of ispE for integration in the cg2 cured region of *C. glutamicum* MB001 | This work |
| pK19mobsacB-Op2 | pK19mobsacB derivative containing the artificial operon dvr_ispGH under the control of the P_tuf promoter with additional ribosome binding sites in front of ispG and ispH for integration in the cg22 cured region of *C. glutamicum* MB001 | This work |
| pVWEx1 | KmR; *E. coli/C. glutamicum* shuttle vector for regulated gene expression (Ptac, lacIq, pCG1 onIVCg) | Peters-Wendisch et al. (2001) |
| pVWEx1-crtB/ | pVWEx1 derivative for IPTG-inducible expression of crtE and the cluster crtB/I from *C. glutamicum* containing artificial ribosome binding sites each | Heider et al. (2014a) |
| pVWEx1-dxs | pVWEx1 derivative for IPTG-inducible overexpression of dxs (cg2083) containing an artificial ribosome binding site in front of the gene | This work |
| pVWEx1-idi | pVWEx1 derivative for IPTG-inducible overexpression of idi (cg2531) containing an artificial ribosome binding site in front of the gene | This work |
| pVWEx1-glpKd | pVWEx1 derivative for IPTG-inducible overexpression of glpF, glpK, and glpD from *E. coli* MG1655 | Rittmann et al. (2008) |
| pKEEx3 | SpecR; *E. coli/C. glutamicum* shuttle vector for regulated gene expression (Ptac, lacIq, pBL1 onIVCg) | Stansen et al. (2005) |

(Continued)
the primer pairs 15/16, 17/18, and 19/20, respectively. An artificial RBS in front of ispG and ispH each was introduced by the oligonucleotides 17 and 19, respectively. Also the genes of Op2 were put under the control of the P\textsubscript{tuf} promoter, amplified from genomic DNA using the primers 13 and 14. Genomic regions flanking the selected insertion region were amplified from genomic DNA of C. glutamicum LYC3 using primer pairs 1/2 and 9/10 for integration in the cgp2 cured region in the case of Op1, or 11/12 and 20/22 for integration of Op2 in the cgp1 cured region (Table 2), respectively. The purified PCR products were either linked by crossover PCR or were directly combined together with the plasmid by Gibson assembly (Gibson et al., 2009). The final assembly of the insert with linearized pEKEx3 or pVWEx1 plasmid DNA. The amplification of the \textit{crt} genes from was based on genomic DNA as template. The amplified products were cloned into the appropriately restricted pEKEx3 or pVWEx1 plasmid DNA.

**EXTRACTION ANALYSIS OF CAROTENOIDS**

To extract carotenoids from the \textit{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\textsubscript{2}O. The pigments were extracted with 10 ml methanolaceton 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).

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 HPLC analysis (see below) and the concentrations were calculated using a standard curve and appropriate dilutions. High performance liquid chromatography (HPLC) analyses of the \textit{C. glutamicum} extracts were performed like described earlier (Heider et al., 2014a) on an Agilent 1200 series HPLC system (Agilent Technologies Sales & Services GmbH & Co., KG, Waldbronn), including a diode array detector (DAD) for UV/visible (Vis) spectrum recording. For separation, a column system consisting of a precolumn (10 mm × 4 mm MultoHigh 100 RP18-5, CS Chromatography Service GmbH, Langerwehe, Germany) and a main column (ProntoSil 200-5 C30, 250 mm × 4 mm, CS Chromatographic Service GmbH, Langerwehe, Germany) was used. Quantification of carotenoids was performed using the extracted wavelength

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**Table 1 | Continued**

| Strain, plasmid | Relevant characteristics | Source or reference |
|----------------|--------------------------|---------------------|
| pEKEx3-crtEbY | pEKEx3 derivative for IPTG-inducible expression of \textit{crtEb} and \textit{crtY} from \textit{C. glutamicum} containing artificial ribosome binding sites in front of each gene | Heider et al. (2014a) |
| pEKEx3-crtY | pEKEx3 derivative for IPTG-inducible expression of \textit{crtY} from \textit{P. ananatis} containing an artificial ribosome binding site in front of the gene | Heider et al. (2014a) |
| pEKEx3-crtYZ | pEKEx3 derivative for IPTG-inducible expression of \textit{crtY} and \textit{crtZ} from \textit{P. ananatis} containing artificial ribosome binding sites in front of each gene | Heider et al. (2014a) |
| pEKEx3-crtYZW | pEKEx3 derivative for IPTG-inducible expression of \textit{crtY} and \textit{crtZ} from \textit{P. ananatis} and \textit{crtW} of \textit{Brevundimonas aurantiaca} containing artificial ribosome binding sites in front of each gene | This work |
| pEKEx3-dxs | pEKEx3 derivative for IPTG-inducible overexpression of \textit{dxs} (cg2083) containing an artificial ribosome binding site in front of the gene | This work |

**PROMOTER EXCHANGE OF THE \textit{dxs} GENE IN \textit{C. GLUTAMICUM} LYC3**

The plasmid pK19mobsacB-P\textsubscript{tuf}d_xs was constructed to replace the native \textit{dxs} promoter with the \textit{tuf} promoter region from \textit{C. glutamicum} WT. For this purpose, the upstream region of \textit{dxs} (483 bp), the 3’ part of \textit{dxs} and the \textit{tuf} promoter region [200 bp upstream of the coding sequence of the \textit{tuf} gene (cg0587)] were amplified from chromosomal DNA of \textit{C. glutamicum} LYC3 using the oligonucleotide pairs 27/28, 23/24, and 25/26, respectively (Table 2). By crossover PCR, the \textit{dxs} 3’ fragment and the \textit{tuf} promoter region were fused with oligonucleotides 23/26. Afterward, the \textit{dxs} upstream region was fused to this 644 bp long fragment using oligonucleotides 27/26. The final purified PCR product was cloned into pK19mobsacB resulting in the vector pK19mobsacB-P\textsubscript{tuf}d_xs (Table 1). The following process for the promoter exchange by two-step homologous recombination was performed as described earlier for the deletion of genes. The promoter exchange was verified by PCR using the primers d_xs_E and 33, and sequencing of the PCR product.

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**OVEREXPRESSION OF CAROTENOGENIC GENES**

Plasmids harboring a carotenogenic gene (general abbreviation \textit{crt}), pEKEx3-crt or pVWEx1-crt allowed an IPTG-inducible overexpression of \textit{crt}. They were constructed on the basis of pEKEx3 (Stansen et al., 2005) or pVWEx1 (Peters-Wendisch et al., 2001), respectively. Amplification of \textit{crt} by polymerase chain reaction (PCR) from genomic DNA of \textit{C. glutamicum} WT, \textit{P. ananatis} and \textit{B. aurantiaca}, which was prepared as described (Eikmanns et al., 1995), was carried out using the respective primers (Table 2). The amplification of the \textit{crt} genes from was based on genomic DNA as template. The amplified products were cloned into the appropriately restricted pEKEx3 or pVWEx1 plasmid DNA.
Table 2 | Oligonucleotides used in this study.

| Oligonucleotide | Sequence (5′ → 3′) |
|-----------------|---------------------|
| **crtEb-A**     | AAAACCGGGACTACCACTCCCGAGGT |
| **crtEb-B**     | CCTCCACCTAATTAAACATGAAATTTTCCATCAT |
| **crtEb-C**     | GCTATGTGGGAGGCTTCGC |
| **crtEb-D**     | AAAACCGGGAGTGTGGAGGCTTCGC |
| **crtEb-E**     | GGAGACTCGGGTATTGTC |
| **crtEb-F**     | AAAACCGGGACTACCACTCCCGAGGT |
| **crtY-A**      | AAAACCGGGACTACCACTCCCGAGGT |
| **crtY-B**      | CATCCACCTAATTAAACATGAAATTTTCCATCAT |
| **crtY-C**      | GCTATGTGGGAGGCTTCGC |
| **crtY-D**      | AAAACCGGGACTACCACTCCCGAGGT |
| **crtY-E**      | CATCCACCTAATTAAACATGAAATTTTCCATCAT |
| **crtY-F**      | GCTATGTGGGAGGCTTCGC |
| **crtZ-w**      | GCTATGTGGGAGGCTTCGC |
| **crtZ-rv1**    | GGAGACTCAGCGTCGACGACAGAATTTTCCATCAT |
| **crtZ-rv2**    | GCTATGTGGGAGGCTTCGC |
| **crtZ-fw2**    | GCTATGTGGGAGGCTTCGC |
| **crtZ-rv**     | GCTATGTGGGAGGCTTCGC |
| **crtW-fw**     | GCTATGTGGGAGGCTTCGC |
| **crtW-rv**     | GCTATGTGGGAGGCTTCGC |

(Continued)
Table 2 | Continued

| Oligonucleotide | Sequence (5′→3′) |
|-----------------|-----------------|
| A1              | CTCGACGCCATCGTCAGAAGGGAGGCCGACCTTCCAGATGCCGGCCCGTCG |
| A2              | CGTTACGGGAGTGCCTATTGTTTTG |
| A3              | CATCACATTAGCCGCGGTCTG |
| A4              | GTGGCGCGGCGAGTCTTGA |
| A5              | CGGTACCCGGGGATC |
| A6              | M13 fw CACAGCGGGAGTGCCTATTGTTTTG |
| A7              | CATCATAACGGTTCTGGC |
| A8              | ATCTTCTCTCATCCGCCA |
| pVWEx-fw        | CACAGCCGGAGTGCCTATTGTTTTG |
| pVWEx-rv        | ATCTTCTCTCATCCGCCA |
| M13 fw          | CACAGCCGGAGTGCCTATTGTTTTG |
| M13 rv          | CACAGCTGATCATCCTCCTGCC |

Sequence in bold: artificial ribosome binding site; sequence underlined: restriction site; sequence in italics: linker sequence for hybridization.

Table 3 | Influence of chromosomal promoter exchange of the 1-deoxy-d-xylulose 5-phosphate synthase gene dxs on Dxs activities, growth rates, and lycopene production.

| C. glutamicum strain | Growth rate (h−1) | final OD (600 nm) | Dxs sp. act. (mU mg−1) | Lycopene production (mg g−1 DCW) |
|----------------------|------------------|-----------------|------------------------|-------------------------------|
| LYC3                 | 0.45 ± 0.01      | 27 ± 1          | 9 ± 1                  | 0.04 ± 0.01                  |
| LYC3-Pdxs            | 0.44 ± 0.02      | 24 ± 2          | 16 ± 1                 | 0.08 ± 0.01                  |
| LYC3pEKEx3(dxs)      | 0.38 ± 0.01      | 22 ± 2          | 26 ± 3                 | 0.06 ± 0.01                  |

Cells were grown in glucose CGXII minimal medium for 24 h. Means and standard deviations of three cultivations are reported.

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), astaxanthin (Ehrenstorfer GmbH, Augsburg, 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 decaprenoxanthin and zeaxanthin 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.

DXS ACTIVITY ASSAY

The DXS activity of C. glutamicum crude extracts was determined using an endpoint assay adopted from Xiang et al. (2007), which is based on the measurement of the remaining pyruvate level in the reaction mixture. The assays were carried out at 30°C in total volume of 1 ml containing 50 mM Tris (pH 7.5), 60 µM pyruvate, 60 µM GAP, 10 mM dithiothreitol (DTT), 5 mM MgCl₂, and 600 µM TPP. Reactions were stopped after 5, 15, 30, and 60 min of incubation by heat inactivation (5 min at 95°C). Subsequent the leftover pyruvate was converted to lactate with lactate dehydrogenase and the concomitant consumption of NADH was determined by fluorescence. Therefore, the reaction was allowed to proceed for 60 min at room temperature. Then, 2.5 µl−1 lactate dehydrogenase and 0.1 mM NADH was added to the reaction mixture and incubated for 30 min at 37°C. The NADH diminution was determined photometrically at 340 nm.

RESULTS

OVEREXPRESSION OF dxs INCREASED LYCOPENE YIELD

The first and often rate-limiting reaction in the MEP pathway is the condensation of pyruvate and GAP to DXP catalyzed by DxS (Harker and Bramley, 1999; Kim and Keasling, 2001). To test if Dxs is a bottleneck in carotenoid biosynthesis in C. glutamicum, dxs was overexpressed in C. glutamicum LYC3, a mutant derived from the genome-reduced C. glutamicum strain MB001 (Baumgart et al., 2013) that accumulates lycopene due to deletion of the lycopene elongase and C45/C50 carotenoid ε-cyclase genes crtEB and cttE. To exchange the native dxs promoter by the strong constitutive promoter of tac (cg0587), which encodes for the elongation factor EF-Tu (Fukui et al., 2011), the replacement vector pK19mobsacB-P_r7dxs was constructed and C. glutamicum LYC3-P_r7dxs was obtained. DxS activities measured in crude extracts were about twofold higher in C. glutamicum LYC3-P_r7dxs (16 ± 1 mU mg⁻¹) than in the control strain C. glutamicum LYC3 (Table 3). As consequence of enhanced Dxs activity, lycopene production doubled (0.08 ± 0.01 mg g⁻¹ DCW as compared to 0.04 ± 0.01 mg g⁻¹ DCW) (Table 3). Thus, increased Dxs activity improved lycopene production by C. glutamicum. Increased specific Dxs activities were also observed when a plasmid-borne copy of dxs was overexpressed from an IPTG-inducible promoter in LYC3, but lycopene production was only slightly improved (Table 3). Hence, chromosomal overexpression proved better and was therefore chosen for subsequent metabolic engineering of the MEP pathway.

OVERPRODUCTION OF ENZYMES CONVERTING DXP TO IPP USING TWO SYNTHETIC OPERONS INTEGRATED INTO THE C. GLUTAMICUM CHROMOSOME

For overproduction of the six MEP pathway enzymes catalyzing the conversion of DXP to IPP, two synthetic operons were...
constructed and integrated into the chromosome of *C. glutamicum* LYC3. Operon 1 was constructed to drive expression of *ispD*, which are cotranscribed naturally, fused to *ispE* from _P_. The RBS of the *tuf* gene was inserted upstream of _ispD_, while the endogenous RBS of _ispE_ and a perfect _C. glutamicum_ RBS upstream of _ispE_ were used. To construct operon 2, _dxr, ispG_, and _ispH_ were fused for expression from _P_. and perfect _C. glutamicum_ RBS were inserted upstream of _ispG_ and _ispH_ while the RBS of the _tuf_ gene was used upstream of _dxr_. Both operons were integrated by homologous recombination into the chromosome of _C. glutamicum_ LYC3, which lacks prophages cg1 and cg2. Operon 1 was integrated into the chromosome of _C. glutamicum_ LYC3 between cg1506 and cg1525, i.e., at the position that harbors prophage cg2 in the _C. glutamicum_ WT, but which is absent from LYC3, and the resulting strain was named LYC3-Op1. Similarly, _C. glutamicum_ LYC3-Op2 was obtained by integrating operon 2 into the chromosome of _C. glutamicum_ LYC3 at the position (between cg1743 and cg1753) that in _C. glutamicum_ WT harbors prophage cg1, but which is absent from LYC3. The constructed _C. glutamicum_ strain LYC3-Op1Op2 contains both operons in the chromosome instead of prophages cg1 and cg2. _C. glutamicum_ LYC3-Op1 showed slightly higher lycopene accumulation than _C. glutamicum_ strains LYC3 and LYC3-Op2. _C. glutamicum_ LYC3-Op2 grew slower than LYC3 and LYC3-Op1. _C. glutamicum_ LYC3-Op1Op2 that harbors both operons also grew slower, but accumulated almost threefold more lycopene than LYC3. Thus, overexpression of MEP pathway genes from two chromosomally integrated synthetic operons improved lycopene production (Figure 2).

**IMPROVED IPP SUPPLY BY CHROMOSOME-BASED ENHANCEMENT OF MEP PATHWAY GENE EXPRESSION**

To combine chromosome-based overexpression of the genes necessary for conversion of DXP to IPP with overproduction of Dxs, the first enzyme of the MEP pathway, the endogenous promoter of chromosomal _dxs_ was exchanged by _P_. in _C. glutamicum_ LYC3-Op1Op2 and the resulting strain was named _C. glutamicum_ LYC3-MEP. Surprisingly, LYC3-MEP showed slower growth on solid as well as in liquid medium. Poor growth in liquid glucose medium was accompanied by little lycopene production, although LYC3-MEP colonies appeared well pigmented on plates. Since the central carbon metabolites pyruvate and GAP are the immediate precursors of the MEP pathway, it was tested if lycopene production by _C. glutamicum_ LYC3-MEP was affected by the carbon source. To this end, pyruvate and glycerol were tested as carbon sources. Since glycerol is no carbon source for _C. glutamicum_ WT, glycerol was induced by 50 mM glucose (Gly/Glu), respectively. Expression of _dxr-ispGH_ operon (LYC3-Op1) showed higher lycopene accumulation than _C. glutamicum_ strains LYC3 and LYC3-Op2. _C. glutamicum_ LYC3-Op1Op2 grew slower than LYC3 and LYC3-Op1. _C. glutamicum_ LYC3-Op1Op2 harbors both operons also grew slower, but accumulated almost threefold more lycopene than LYC3. Thus, overexpression of MEP pathway genes from two chromosomally integrated synthetic operons improved lycopene production (Figure 2).

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**FIGURE 2 | Lycopene production by *C. glutamicum* LYC3 and derived strains expressing the synthetic _ispD-ispE_ operon (LYC3-Op1), the synthetic _dxr-ispGH_ operon (LYC3-Op2) or both operons (LYC3-Op1Op2) for overproduction of MEP pathway enzymes.** Cells were grown in glucose CGXII minimal medium. Means and standard deviations of three cultivations are shown.

**FIGURE 3 | Lycopene production by *C. glutamicum* LYC3-MEP(pVWE1-1-glPFK) on glycerol as sole and combined carbon source.** Cells were grown in CGXII minimal medium with 200 mM glycerol (Gly), 100 mM glycerol + 100 mM pyruvate (Gly/Pyr), or 100 mM glycerol + 50 mM glucose (Gly/Glu), respectively. Expression of _glPFK_ was induced by 50 µM IPTG. As reference, lycopene production of the strains LYC3 and LYC3-MEP grown in CGXII minimal medium with 100 mM glucose (Glu) is given. Means and standard deviations of three cultivations are shown.
growth and carotenogenesis. To test this hypothesis, isopentenyl pyrophosphate isomerase  

Idi was overproduced. Indeed, *C. glutamicum* LYC3-MEP(pVWEX1-id)(pEKEx3) produced twofold more lycopene (0.08 ± 0.02 mg g⁻¹ DCW) than *C. glutamicum* strains LYC3, LYC3-MEP, and the empty vector control strain, but still showed impaired growth (Table 4). Thus, a lycopene producing *C. glutamicum* strain with improved IPP supply overexpressing all MEP pathway genes and idi could be constructed. However, lycopene production by this strain (Table 4) was comparable to that by *C. glutamicum* strains LYC3-P1ufdsx (Table 3) and LYC3-Op10p2 (Figure 2) indicating that the positive effects did not act synergistically. This was also observed when the strains were grown in LB medium supplemented with 100 mM glucose; however, they grew faster (data not shown). Taken together, *C. glutamicum* strains with improved IPP and DMAPP supply showed higher lycopene production than the respective parental strains.

**APPLICATION OF C. GLUTAMICUM WITH IMPROVED IPP SUPPLY FOR PRODUCTION OF DECAPRENOXANTHIN AND ASTAXANTHIN**

To test if *C. glutamicum* LYC3-MEP overexpressing idi is suitable for production of the endogenous C50 carotenoid decaprenoxanthin, this strain was transformed with plasmid pEKEx3-crtEbY. Expression of lycopene elongase gene *crtEb* and of carotenoid ε-cyclase gene *crtYε* from this plasmid complements the lycopene producing *C. glutamicum* LYC3-MEP, which carries chromosomal *crtEb* and *crtYε* deletions allowing for decaprenoxanthin biosynthesis. The resulting strain LYC3-MEP(pVWEX1-id)(pEKEx3-crtEbY) overproduces all enzymes of endogenous carotenogenesis except *crtE*, *crtB*, and *crtl* (Figure 1). Although it grew slowly, LYC3-MEP(pVWEX1-id)(pEKEx3-crtEbY) produced 0.35 ± 0.02 mg g⁻¹ DCW (Table 5) and, thus, is a genome-reduced strain with improved IPP supply suitable for the overproduction of the endogenous C50 carotenoid decaprenoxanthin.

*C. glutamicum* has previously been engineered for the production of the non-native C40 carotenoids β-carotene and zeaxanthin (Heider et al., 2014a). When *crtYPα* (PANA_4160) encoding lycopene cyclase from *Pantoea ananatis* was expressed, β-carotene accumulated. Additional expression of *crtZPα* (PANA_4163), which encodes β-carotene hydroxylase, resulted in partial conversion of β-carotene to zeaxanthin (Heider et al., 2014a). To enable astaxanthin production, *crtWβα* encoding carotene C(4) oxygenase from *Brevundimonas aurantiaca*, which oxidizes zeaxanthin to yield astaxanthin, was expressed in addition to *crtYPα* and *crtZPα*. The resulting plasmid pEKEx3-crtZWy was used to transform LYC3-MEP(pVWEX1-id)(pEKEx3-crtEbY) to produce 0.14 ± 0.01 mg g⁻¹ DCW astaxanthin and neither β-carotene nor zeaxanthin accumulated (Table 5). Thus, to the best of our knowledge, this is the documentation of astaxanthin production by recombinant *C. glutamicum*. Although levels were low, LYC3-MEP(pVWEX1-id)(pEKEx3-crtZWy) produced astaxanthin as only carotenoid.

Based on our previous findings that overexpression of the genes *crtE*, *crtB*, and *crtl* (Figure 1) strongly increased lycopene production (Heider et al., 2012), as well as decaprenoxanthin production (Heider et al., 2014a); these genes were overexpressed from plasmid pVWEX3-crtEbI. The resulting strain *C. glutamicum* LYC3-MEP(pVWEX3-crtEbI)(pEKEx3-crtZWy) produced 2.1 ± 1.3 mg g⁻¹ DCW β-carotene and 1.2 ± 0.2 mg g⁻¹ DCW zeaxanthin (Table 5), but also ninefold more astaxanthin (1.2 ± 0.5 mg g⁻¹ DCW) than LYC3-MEP(pVWEX1-id)(pEKEx3-crtZWy). Thus, it was shown that astaxanthin can be produced by recombinant *C. glutamicum* in the milligrams per gram DCW range.

**DISCUSSION**

Recently, *C. glutamicum* has been engineered for production of diverse lycopene-derived carotenoids (Heider et al., 2014a) and of a sesquiterpene (Frohwitter et al., 2014). There is an increasing demand for efficient, low-cost, and natural production of terpenoids (Zhu et al., 2014) as they have many applications,
e.g., in the medicinal and nutraceutical industries or as fuels (Martin et al., 2003; Ajiikumar et al., 2010; Peralta-Yahya et al., 2011). Besides terminal terpenoid pathway engineering, an efficient supply of the prenyl pyrophosphate precursors is important (Heider et al., 2014b). It could be shown here that MEP pathway engineering to improve IPP supply in *C. glutamicum* improved lycopene production. However, as observed in similar studies of MEP pathway engineering in other bacteria individual bottlenecks may be overcome, but the individual beneficial effects do not necessarily add up (Kim and Keasling, 2001; Martin et al., 2003; Rodriguez-Villalon et al., 2008). Overexpressing the initial MEP pathway gene, *dxs* improved lycopene production by *C. glutamicum* (see Figure 1) and by other bacteria (Harker and Bramley, 1999; Matthews and Wurtzel, 2000). However, optimal overexpression levels need to be established since, e.g., chromosomal overexpression proved better than overexpression from a mutly-copy plasmid (Yuan et al., 2006). Similarly, when *dxs* was overexpressed in *C. glutamicum* by exchanging the native promoter of *dxs* with the strong constitutive tuf promoter more lycopene accumulated than when plasmid-borne *dxs* overexpression, which led to higher Dxs activities, was tested (Table 3). The complex interplay of MEP pathway enzymes is also reflected by the fact that overexpression of *dxr*, *ispG*, and *ispH* in LYC3-Op2 only improved lycopene accumulation when combined with overexpression of *ispDF* and *ispE* (Op1) (Figure 2). Although lycopene titers obtained with *C. glutamicum* LYC3-Op1Op2 were comparable to the *dxs* overexpressing strain LYC3-Op1dxs (Figure 2 and Table 3), their combination in strain LYC3-MEP was not synergistic and even perturbed growth. This may be explained by accumulation of inhibitory MEP pathway intermediates as shown for *B. subtilis* (Sivy et al., 2011) and *E. coli* (Martin et al., 2003; Zou et al., 2013), from an excessive drain of central metabolic intermediates (Kim and Keasling, 2001) and/or from an imbalance between IPP and DMAPP (Kajiwara et al., 1997). In *C. glutamicum*, improved lycopene production as consequence of overexpression of IPP isomerase gene *idi* was observed in LYC3-MEP (Table 4). However, lycopene production by LYC3-MEP overexpressing *idi* was not higher than by LYC3-P_n3dxs or by LYC3-Op1Op2. Moreover, when *dxs* was overexpressed in the WT-derived strain ΔcrtEb lycopene production increased from about 0.04 to about 0.12 mg g⁻¹ DCW, but combined overexpression of *dxs* and *idi* did not further increase lycopene production (data not shown). Thus, the perturbed growth may not only be due to an imbalance between IPP and DMAPP.

It remains to be shown if combinatorial approaches to optimize multiple gene expression levels (Zelbuch et al., 2013; Nowroozi et al., 2014) would improve the IPP precursor supply in *C. glutamicum*. Fine-tuning of gene expression in recombinant *C. glutamicum* by varying promoters (Holátko et al., 2009; van Ooyen et al., 2011; Schneider et al., 2012), RBSs (Schneider et al., 2012), translational start codons (Schneider et al., 2012), or translational stop codons (Jensen and Wendisch, 2013) improved production of amino acids and diamines. In addition, overexpression of heterologous instead of endogenous genes may be beneficial, e.g., as shown for improving isoprene production by *E. coli* via overexpression of two MEP pathway genes *dxs* and *dxr* from *B. subtilis* (Zhao et al., 2011) or by combining overexpression of *xylA* from *Xanthomomas campestris* with endogenous *xylB* to accelerate xylose utilization of *C. glutamicum* (Meiwinkel et al., 2013).

Besides fine-tuning of MEP pathway gene overexpression, growth, and terpenoid production by recombinant *C. glutamicum* with increased IPP supply could be improved by metabolic pull, i.e., by overexpression of genes of the downstream terpenoid pathway (Table 5). Similarly, amorphadiene synthase overexpression prevented accumulation of inhibitory isoprenoid pathway intermediates in *E. coli* (Martin et al., 2003). Overcoming the toxicity of accumulating IPP and DMAPP was successfully used as screening method for the identification of genes that are involved in isoprenoid biosynthesis (Withers et al., 2007). Accumulation of the MEP pathway intermediate ME-cPP inhibits growth and isoprenoid production by recombinant *E. coli*. To abolish its accumulation overproducing the two enzymes downstream of ME-cPP (*ispG* and *ispH*) needed to be combined with overexpressing an operon for iron–sulfur cluster assembly since both IspG and IspH are containing iron–sulfur clusters (Zou et al., 2013).

To the best of our knowledge, production of astaxanthin by recombinant *C. glutamicum* was shown here for the first time. Astaxanthin is the third most important carotenoid after β-carotene and lutein and its global market amounted to about 230 million US$ in 2010 (BBC Research, 2011). The economically most significant application of astaxanthin is its use as feed additive in aquaculture industry (Lorenz and Cysewski, 2000; Higuera-Ciapara et al., 2006; Schmidt et al., 2011), but it also exhibits high potential as a nutraceutical and as an approved ingredient for cosmetics due to its remarkably high antioxidative activity (Miki, 1991; Schmidt et al., 2011). Astaxanthin is mainly produced by marine bacteria and microalgae, but only the green freshwater microalga *Haematococcus pluvialis* and the red yeasts *Xanthophyllomyces dendraohous*/*Phaffia rhodozyma* are established as hosts for commercial production (Bhosale and Bernstein, 2005; Rodriguez-Saiz et al., 2010). Algae-based production of astaxanthin is still more costly than chemical synthesis (Jackson et al., 2008), but markets more and more demand naturally produced carotenoids. The astaxanthin titers by recombinant *C. glutamicum* reported here are in the milligrams per gram DCW range and, thus, they are comparable to yields described for *P. rhodozyma* (ranging from 0.16 to 6.6 mg g⁻¹ DCW (Cruz and Parajo, 1998; Jacobson et al., 1999). The highest product titer of 9.7 mg g⁻¹ DCW is reported for a *P. rhodozyma* strain improved by metabolic engineering and classical mutagenesis (Gassel et al., 2013), while the highest titer in a recombinant bacterium, i.e., *E. coli* strain was 5.8 mg g⁻¹ DCW astaxanthin (Zelbuch et al., 2013). Thus, the astaxanthin titers reported for *C. glutamicum* are comparable and it is conceivable that they may be improved further by combining metabolic engineering with classical mutagenesis as in *P. rhodozyma* (Gassel et al., 2013), by combinatorial approaches to gene expression (Zelbuch et al., 2013), or by high-cell density cultivation since biomass concentrations of up to 95 g DCW/l have been reported for *C. glutamicum* (Riesenberg and Guthke, 1999).

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