Isoprenoid Pyrophosphate-Dependent Transcriptional Regulation of Carotenogenesis in Corynebacterium glutamicum

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Corynebacterium glutamicum is a natural producer of the C50 carotenoid decaprenoxanthin. The crtEcgc0722crtBIYEb operon comprises most of its genes for terpenoid biosynthesis. The MarR-type regulator encoded upstream and in divergent orientation of the carotenoid biosynthesis operon has not yet been characterized. This regulator, named CrtR in this study, is encoded in many actinobacterial genomes co-occurring with terpenoid biosynthesis genes. CrtR was shown to repress the crt operon of C. glutamicum since DNA microarray experiments revealed that transcript levels of the crt operon were increased 10 to 70-fold in its absence. Transcriptional fusions of a promoter-less gfp gene with the crt operon and crtR promoters confirmed that CrtR represses its own gene and the crt operon. Gel mobility shift assays with purified His-tagged CrtR showed that CrtR binds to a region overlapping with the −10 and −35 promoter sequences of the crt operon. Isoprenoid pyrophosphates interfered with binding of CrtR to its target DNA, so far unknown mechanism for regulation of carotenogenesis. The molecular details of protein-ligand interactions remain to be studied. Decapreno-faxanthin synthesis by C. glutamicum wild type was enhanced 10 to 30-fold upon deletion of crtR and was decreased 5 to 6-fold as result of crtR overexpression. Moreover, deletion of crtR was shown as metabolic engineering strategy to improve production of native and non-native carotenoids including lycopene, β-carotene, C.p. 450 and sarcinaxanthin.

Keywords: regulation of carotenogenesis, terpenoid biosynthesis, actinobacteria, isoprenoid pyrophosphates as inducers, CrtR, MarR-type regulators

INTRODUCTION

Carotenoids, natural yellow to red colored pigments, are the colorful representatives of the versatile and extensive group of terpenoids. Carotenoids have diverse biological functions serving for instance as photo protectors, light harvesting molecules or as membrane stabilizers (Sandoval et al., 1984; Lee and Schmidt-Dannert, 2002). They are synthesized by plants, fungi, algae and bacteria (Britton et al., 2004). Commercially, carotenoids and their derivatives are mainly applied in the
food, beverage and cosmetics industries (Downham and Collins, 2000; Winterhalter and Roussef, 2001; Dembitsky, 2005), but they are also receiving increasing attention due to their potential beneficial effects on human health (Armstrong, 1994; Sandmann, 2001; Umeno et al., 2005; Das et al., 2007). The total commercial market value of carotenoids was $1.5 billion in 2014 and it is expected to increase to $1.8 billion in 2019 (BBCResearch, 2015).

*Corynebacterium glutamicum*, a GRAS organism used in industrial biotechnology, is a natural producer of the C50 carotenoid decaprenoxanthin, responsible for its characteristic yellow pigmentation. *C. glutamicum* belongs to a rare group of bacteria producing long chain carotenoids. Carotenogenesis is not essential in *C. glutamicum* since transposon mutants (Krubasik et al., 2001a;b; Sandmann and Yukawa, 2005) as well as directed gene deletion mutants were shown to be deficient of carotenoid biosynthesis, but viable (Heider et al., 2012). C50 carotenoids have been mainly isolated from extremely halophilic archaea (Kelly and Jensen, 1967; Pfander, 1994) and from Gram-positive bacteria of the order Actinomycetales (Netzer et al., 2010). The pathway for decaprenoxanthin biosynthesis starting from the precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and the respective carotenogenic genes of *C. glutamicum* are characterized (Krubasik et al., 2001a; Heider et al., 2012). Besides the biosynthesis of the ε-cyclic decaprenoxanthin in *C. glutamicum* only four other pathways toward C50 carotenoids have been elucidated: the pathway of the β-cyclic C.p.450, first isolated from *C. poinsettiae* (Norgård et al., 1970), in *Dietzia sp*. CQ4 (Tao et al., 2007), the γ-cyclic C50 carotenoid sarcinaxanthin in *Micrococcus luteus* NCTC2665 (Netzer et al., 2010) and recently the route to the acyclic bacterioruberin in the extremely halophilic archaeon *Halocarcula japonica* was described (Yang et al., 2015).

In many bacteria the carotenogenic genes are clustered. *C. glutamicum* possesses the crt operon with seven co-transcribed genes (crtE, cg0722, crtB, ctcR, crtYc, ctcYf, ctcYb) necessary for the conversion of IPP and DMAPP to decaprenoxanthin, and one gene of unknown function (cg0722). A second functional phytoene synthase is encoded elsewhere in the genome (Heider et al., 2012). The major geranylgeranyl pyrophosphate (GGPP) synthase of *C. glutamicum* is not encoded by crtE, the first gene of the crt operon, but by idsA that is located elsewhere on the *C. glutamicum* chromosome (Heider et al., 2014a). It is not known whether synthesis of the redundant GGPP synthase and phytoene synthase enzymes is regulated.

Biosynthesis of terpenoids is commonly triggered by photooxidative stress factors in plant, fungi and bacteria (Bramley and Mackenzie, 1988), as they serve e.g., as light harvesting molecules or as protection against excess light energy or against the resulting reactive oxygen species (Johnson and Schroeder, 1996; Britton, 2008). Little is known about the regulatory mechanisms governing carotenoid biosynthesis in bacteria which may change in response to blue light, oxygen levels or growth phase (Johnson and Schroeder, 1996). The mechanism of light-induced carotenogenesis in the non-photosynthetic Gram-negative bacterium *Mycococcus xanthus* (Fontes et al., 2003), the Gram-positive bacteria *Streptomyces coelicolor* A3(2) (Takano et al., 2005), *Thermus thermophilus* (Takano, 2016) and *Bacillus megaterium* (Takano et al., 2015b) has been studied in more detail. MerR-type transcriptional regulators with a vitamin B12 binding domain are involved in regulation of carotenogenesis in those organisms. In addition, the RNA polymerase sigma factor SigF has been shown to be involved in regulation of carotenoid biosynthesis in *Mycobacterium smegmatis* (Provvedi et al., 2008).

Regulation of carotenoid biosynthesis in *C. glutamicum* has not been investigated so far. Gene expression analyses performed under different stress conditions revealed that mRNA levels of *crtE*, the first gene of the *crt* operon, altered in response to various stresses (Silberbach et al., 2005; Feldmann et al., 2009; Jochmann et al., 2009). The putative multiple antibiotic resistance (MarR)-family transcriptional regulator gene cg0725, named *crtR* due to the results described in this study, is located upstream of the *crt* operon and is transcribed in divergent orientation to the *crt* operon. Previously, it has been shown that *crtR* was disrupted by transposon insertion in a mutant accumulating 3-fold more decaprenoxanthin (Krubasik et al., 2001a), but the regulatory mechanism has not been characterized. The majority of characterized MarR proteins are transcriptional repressors and their genes are generally found in the vicinity or are part of the regulated gene cluster (Wilkinson and Grove, 2006). This genetic organization and the finding that transposon insertions into this gene and the orthologous gene in the related *Mycobacterium marinum* increased biosynthesis of decaprenoxanthin and β-carotene (Krubasik et al., 2001a; Gao et al., 2003), prompted us to characterize CrtR with respect to regulation of carotenogenesis in *C. glutamicum*.

## MATERIALS AND METHODS

### Bacterial Strains, Media, and Growth Conditions

The strains and plasmids used in this work are listed in Table 1. *C. glutamicum* ATCC 13032 was used as wild type (WT) and the prophage cured *C. glutamicum* MB001 (Baumgart et al., 2013) as basal strain for genetic engineering. *C. glutamicum* cultivations were performed in CGXII medium with 100 mM glucose as carbon and energy source (Eggeling and Reyes, 2005) after inoculation to an initial OD of 50 ml in 500 ml flasks with two baffles shaking at 120 rpm or in 1 ml volume in microtiterplates at 1100 rpm at 30°C using Biolector® micro fermentation system (m2p-labs GmbH, Baesweiler, Germany). The growth was followed by measuring the OD660 using a Shimadzu UV-1202 spectrophotometer (Duisburg, Germany). Cloning was conducted with *E. coli* DH5α as host, cultivated in LB medium at 37°C. When appropriate, kanamycin (25 μg/ml), spectinomycin (100 μg/ml), or ampicillin (100 μg/ml) was added to the culture medium. If not stated otherwise 1 mM of IPTG was added for induction of gene expression 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 NucleoSpin kit (MACHEERY-NAGEL GmbH & Co. KG, Düren, Germany). Oligonucleotides used in this
TABLE 1 | Strains, plasmids and oligonucleotides used in this study.

| Strain, plasmid or oligonucleotide | Relevant characteristics or sequence | References |
|------------------------------------|-------------------------------------|------------|
| **E. coli STRAINS**                |                                     |            |
| DH5α                               | F− thi−1 endA1 hsdR17(r− m−) supE44 ΔlacU169 (Δ80lacZΔM15) recA1 gyrA96 relA1 | Hanahan, 1983 |
| BL21 (DE3)                         | F− ampT hsdS2F(rK− mK−) recA3 deoR gal dcm (DE3) | Novagen    |
| **C. glutamicum STRAINS**          |                                     |            |
| WT                                 | ATCC 13032                          | Abe et al., 1967 |
| WT ΔcrtR                           | cg0725 (crtR) deletion mutant of C. glutamicum ATCC 13032 | this work |
| MB001                              | ATCC 13032 with in-frame deletion of prophages cg1 (cg1507-cg1524), cg2 (cg1746-cg1752), and cg3 (cg1890-cg2071) | Baumgart et al., 2013 |
| MB001 ΔcrtR                        | cg0725 (crtR) deletion mutant of C. glutamicum MB001 | this work |
| Lyc5                               | crtYc YcYb deletion mutant of MB001 with artificial operon containing cte, cteB and cte under control of the Ptef promoter integrated into the chromosome and chromosomal promoter exchange of dxs (Pdxs) | Henke et al., 2016 |
| Lyc5 ΔcrtR                         | cg0725 (crtR) deletion mutant of C. glutamicum Lyc5 | this work |
| BETA3                              | Lyc5 derivative with crtYcYb under control of the Ptef promoter integrated into the chromosome | Henke et al., 2016 |
| BETA3 ΔcrtR                        | cg0725 (crtR) deletion mutant of C. glutamicum BETA3 | this work |
| **PLASMIDS**                       |                                     |            |
| pK19mob sacB                       | KmR; E. coli/C. glutamicum shuttle vector for construction of insertion and deletion mutants in C. glutamicum (pK18 oriV<sub>E.c.</sub> sacB lacZ<sub>B</sub>) | Schäfer et al., 1993 |
| pK19mob sacB-crtR                  | pK19mob sacB with a cg0725 deletion construct | Henke et al., 2016 |
| pWEx1                              | KmR; E. coli/C. glutamicum shuttle vector for regulated gene expression (P<sub>Eac-lacR</sub>; pCG1 oriV<sub>C.g.</sub>) | Peters-Wendisch et al., 2001 |
| pWEx1-crtR                         | pWEx1 derivative for IPTG-inducible expression of cg0725 from C. glutamicum containing an artificial ribosome binding site in front of the gene | this work |
| pEKEx3                             | Spec<sup>R</sup>; E. coli/C. glutamicum shuttle vector for regulated gene expression (P<sub>Eac-lacR</sub>; pBL1 oriV<sub>C.g.</sub>) | Stansen et al., 2005 |
| pEKEx3-crtE2Y                      | pEKEx3 derivative for IPTG-inducible expression of crtE2Y from M. luteus | Heider et al., 2014b |
| pEKEx3-ibtABC                      | pEKEx3 derivative for IPTG-inducible expression of ibtABC from Dietzia sp. CQ4 | Heider et al., 2014b |
| pEPR1                              | Kan<sup>R</sup>; vector for transcriptional fusion analysis with the promoter-less gfp<sub>uv</sub> reporter gene | Knoppova et al., 2007 |
| pEPR1-PortE                        | pEPR1 derive with 5′UTR region of cte | this work |
| pEPR1-PortE_M1<sup>*</sup>         | pEPR1 derive with 5′UTR region of cte with mutation in motif 1 (TTAA to GGCC) | this work |
| pEPR1-PortE_M2<sup>*</sup>         | pEPR1 derive with 5′UTR region of cte with mutation in motif 2 (AAATT<sub>T</sub> to CCGGG) | this work |
| pEPR1-PortE_M12<sup>*</sup>        | pEPR1 derive with 5′UTR region of cte with mutation in motif 1 (TTAA to GGCC) and 2 (AAATT<sub>T</sub> to CCGGG) | this work |
| pEPR1-PortR                        | pEPR1 derive with 5′UTR region of cte <sup>T</sup> | this work |
| pEPR1-PortR_M1<sup>*</sup>         | pEPR1 derive with 5′UTR region of cte with mutation in motif 1 (TTAA to GGCC) | this work |
| pEPR1-PortR_M2<sup>*</sup>         | pEPR1 derive with 5′UTR region of cte with mutation in motif 2 (AAATT<sub>T</sub> to CCGGG) | this work |
| pEPR1-PortR_M12<sup>*</sup>        | pEPR1 derive with 5′UTR region of cte with mutation in motif 1 (TTAA to GGCC) and motif 2 (AAATT<sub>T</sub> to CCGGG) | this work |
| pET16b                             | Amp<sup>R</sup>; overproduction of decahistidine tagged proteins in E. coli (pBR322 ori<sub>Ec</sub>. lacI<sub>pl</sub>, lacI<sub>C.g.</sub>) | Novagen |
| pET16b-crtR                       | pET16b derivative for the purification of His-tagged CrtR of C. glutamicum from E. coli BL21(DE3) | this work |
| **OLIGONUCLEOTIDES**               |                                     |            |
| cte-Ndel−<sub>flw</sub>           | AAAACATGCTGTAATATGCGAGGAACC         |            |
| cte-BamH<sub>I</sub>-<sub>rv</sub> | AAAAGGATCCCTGACCTGCCGCGATG         |            |
| SFT3                               | ATTCAGCATGTAATACGCTCT              |            |
| SFT4                               | CAAAATAATGTGCTTAC                 |            |
| SFT8                               | TGGAGTATCAGACCGGCA                |            |
| SFT10                              | AACTCATGAGGACTAAATATTTCC          |            |
| SFT12                              | AAAATATTAAATCTAGGAGGACT            |            |

(Continued)
study were obtained from Eurofins MWG Operon (Ebersberg, Germany) and Metabion (Planegg/Steinkirchen, Germany) and are listed in Table 1. Plasmid construction was executed by standard PCR, restriction and ligation reactions as described previously (Sambrook and Russell, 2001) as well as Gibson assembly (Gibson et al., 2009). The RbCl method was used for transformation of E. coli (Hanahan, 1983) and C. glutamicum was transformed via electroporation at 2.5 kV, 200 /Ω, and 25 µF (Eggeling and Reyes, 2005). The correctness of the cloned DNA fragments was verified by sequencing.

Homologous Overexpression of Genes from C. glutamicum

Plasmids for inducible gene expression were constructed on the basis of pEKEx3 (Stansen et al., 2005) or pVWEx1 (Peters-Wendisch et al., 2001). The gene cfr (cg0725) was amplified from genomic DNA of C. glutamicum WT using the primer pairs cfr_A/B and cfr_C/D (Table 1). The PCR products were purified and assembled and simultaneously cloned into Smal restricted pK19mobsacB by Gibson assembly, which resulted in the deletion vector pK19mobsacB-cfr (Table 1). Targeted gene deletion proceeds via two-step homologous recombination as described previously using the before mentioned deletion vector (Eggeling and Bott, 2005). The first recombination event, the integration of the vector into one of the gene flanking regions, was selected via kanamycin resistance. Integration of the deletion vector into the genome triggers sucrose sensitivity due to the expression of sacB, encoding a levansucrase. In a second recombination event the deletion vector is excised and clones can be selected upon sucrose-resistance. By PCR analysis of three selected mutants using the primer pair cfr_E/F, deletion of the respective gene was verified (Table 1). The PCR products were then sequenced and in-frame deletion of cfr was confirmed.

Extraction of Carotenoids from Bacterial Cell Cultures

The extraction of carotenoids from C. glutamicum was performed as described previously (Heider et al., 2014b) using 0.8 or 1 ml
aliquots of the cell cultures. The pigments were extracted from the cell pellets with a methanol:acetone mixture (7:3) at 60°C for 30 min with thorough vortexing every 10 min. When necessary, extraction was repeated to remove all visible colors from the cell pellet. Subsequently, extraction mixtures were centrifuged for 5 min at 13,000 × g and the clear supernatant was used for analysis.

**Analysis of Carotenoids**

The carotenoid content of cell extracts was determined through absorbance at 470 nm by high performance liquid chromatography (HPLC) analysis as described previously (Heider et al., 2014b). HPLC analyses were performed on an Agilent 1200 series HPLC system (Agilent Technologies GmbH & Co. KG, Böblingen, Germany), including a diode array detector (DAD) for UV/visible (Vis) spectrum recording for detection. Separation of the carotenoids was accomplished by application of a column system (all columns from CS Chromatographie Service GmbH, Langerwehe, Germany) consisting of a pre-column (10 × 4 mm MultoHigh 100 RP18-5) and a main column (ProntoSil 200-5 C30, 250 × 4 mm). Alternatively, 50 µL of the sample was separated with a column system consisting of a precolumn (LiChrospher 100 RP18 EC-5, 40 × 4 mm) and a main column (LiChrospher 100 RP18 EC-5, 125 × 4 mm) with methanol (A) and methanol/water (9:1) (B) as mobile phase. The following gradient was used at a flow rate of 1.5 mL/min: 0 min B: 0%, 10 min B: 100%, 32.5 min B: 100%. Quantification of lycopene and β-carotene was performed running a standard curve with HPLC grade standards (Sigma-Aldrich, Taufkirchen, Germany). Due to the lack of appropriate standards for decaprenoxanthin, sarcinaxanthin and C.p. 450 the standards were dissolved in chloroform according to its solubility and diluted in methanol:acetone (7:3) for generating a standard curve.

**Transcriptome Analysis**

For transcriptome analysis triplicate cultures were grown in LB or CgXII medium. At an optical density of OD₆₀₀ between 4 and 5 cells were harvested and total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. DNA was degraded in two reactions, in solution as well as on-column, using the RNase-Free DNase set (Qiagen, Hilden, Germany). Fluorescently labeled cDNA from 20 µg of total RNA was generated by indirect labeling as described by Hüser et al. (2003). cDNA probes labeled with Cy3 and Cy5 were combined for hybridization. Hybridization and scanning was performed as described earlier (Hüser et al., 2003). Signal-background segmentation, spot finding and intensity quantification were executed by ImaGene 6.0 software (BioDiscovery, Hawthorne, United States). Normalization (lowess) and t-test statistics were accomplished with EMMA 2.2, a software platform for consistent storage and efficient analysis of microarray data (Dondrup et al., 2003, 2009). Genes were regarded as differentially expressed when transcript abundance changed 2-fold or more (M ≥ 1 or M ≤ −1) with a statistical significance of p ≤ 0.05 in a student’s t-test was determined.

**In vivo Measurement of Promoter Activities of crtR and crtE**

In vivo expression levels of crtR and crtE were assayed by transcriptional fusion of the intergenic region of crtR and crtE to the promoter-less GFPUV gene using the pEPR1 plasmid system (Knoppova et al., 2007). Therefore, the corresponding promoter sequences were amplified from genomic DNA of C. glutamicum WT with primer pairs PcrtE-pEPR-fw/PcrtE-pEPR-rv and PcrtR-pEPR-fw/PcrtR-pEPR-rv, respectively and cloned into the BamHI restricted pEPR1 vector upstream of the promoter-less gfpUV gene. Site-directed mutagenesis of putative motifs was performed on both constructed vectors using the primer pairs SDM-fw-nat/ SDM-rv-mut, SDM-fw-mut/ SDM-rv-nat and SDM-fw-mut/SDM-rv-mut. The GFPUV fluorescence was performed on a FACS Gallios™ (Beckmann Coulter GmbH, Krefeld, Germany) with 405 nm excitation from a blue solid-state laser. Forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) were detected as small- and large-angle scatters of the 405 nm laser. GFPUV fluorescence was detected using a 500/50 nm band-pass filter. C. glutamicum MB001 ΔcrtR and MB001 harboring the plasmids pEPR1-PcrtR and pEPR1-PcrtE or the plasmid with the mutated promoter sequences, were harvested in mid-exponential growth washed once in phosphate-buffered saline and the optical density was adjusted to OD₆₀₀ < 1. Cells harboring the empty vector pEPR1 were used to determine background fluorescence.

**Overproduction and Purification of the Transcriptional Regulator CrtR**

The vector pET16b-crtR was constructed using PCR-amplified crtR using oligonucleotides crtR-NdeI-fw and crtR-BamHI-rv and ligating it into pET16b restricted with BamHI and NdeI. E. coli BL21(DE3) cells carrying the plasmid pET16b-crtR were grown at 37°C in 500 ml LB medium with 100 µg/ml ampicillin to an OD₆₀₀ of 0.5 before adding IPTG for induction of the gene expression to a final concentration of 0.5 mM. After induction, cells were cultivated at 21°C for additional 5 h. Cells were harvested by centrifugation and stored at −20°C. For cell extract preparation, thawed cells were re-suspended in TNI buffer (20 mM Tris-HCl, pH 7.9, 300 mM NaCl, 5% (v/v) glycerol) with 5 mM imidazole containing a proteinase inhibitor cocktail tablet (Complete Mini, Roche, Basel, Switzerland). Cells were disrupted by ultrasonification using Hielscher UP200S2 (Teltow, Germany) with an amplitude of 60% and a pulsing cycle of 0.5 (power discharge 0.5 s, pause 0.5 s) for 2 min. After ultracentrifugation (1.5 h, 45,000 × g, 4°C) the supernatant was filtered through a 0.2 µm filter and purified by nickel affinity chromatography using nickel-activated nitrilotriacetic acid-agarose (NTA) (Novagen, San Diego, USA). TNI buffer containing 5 mM or 100 mM imidazole was used for sequentially
Electrophoretic Mobility Shift Assay (EMSA)

For verification of a physical Protein-DNA interaction between the regulator CrtR and the promoter region of \textit{crtE} and \textit{crtR}, band shift assay was performed according to Krause et al. (2012). His-tagged CrtR in varying molar excess was mixed with 90 ng of purified promoter-fragments of the target genes in band shift (BS) buffer (50 mM Tris–HCl, 10% (v/v) glycerol, 50 mM KCl, 10 mM MgCl\textsubscript{2}, 0.5 mM EDTA, pH 7.5) in a total volume of 20 µL. The 5′UTR of \textit{crtE} was PCR-amplified with the oligonucleotides SFT8 and SFT3 (fragment A) listed in Table 1 and purified with NucleoSpin kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Truncated and mutated promoter fragments were amplified using oligonucleotides pairs SFT8/SFT10 (fragment B), SFT8/SFT12 (fragment C), SFT4/SFT27 (fragment D), SFT28/SFT27 (fragment E), SFT29/SFT27 (fragment F), SFT30/SFT27 (fragment G), SFT8/SFT23 (fragment H), SFT8/SFT26 (fragment I), SFT8/SFT25 (fragment K). A 78 bp fragment of the upstream region of \textit{cg2228} was added in every sample as a negative control using oligonucleotides \textit{cg2228-fw} and \textit{cg2228-rv}. After 30 min of incubation at room temperature, gel shift samples were separated on a 6% DNA Retardation gel (Life Technologies GmbH, Darmstadt, Germany) at 100 V buffered in 44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA at pH 8.3. Additionally, the binding affinity in presence of selected effectors was analyzed by incubation of the protein with the effector substance under buffered conditions for 15 min at room temperature prior to the addition of the promoter. Subsequently, the gel shift samples were separated on a 6% DNA Retardation gel (Life Technologies GmbH, Darmstadt, Germany) at 100 V buffered in 44.5 mM Tris, 44.5 mM boric acid, and 1 mM EDTA at pH 8.3. Staining of the DNA was achieved with ethidium bromide.

RESULTS

Co-occurrence of the MarR-Type Regulator Gene \textit{crtR} with Genes of Carotenoid Biosynthesis in Completely Sequenced Actinobacterial Genomes

In the genome of \textit{C. glutamicum} the carotenogenic genes responsible for conversion of the building blocks IPP and DMAPP to decaprenoxanthin are organized in the \textit{crt} operon (\textit{crtE-cg0722-crtBIYcYcEb}) (Heider et al., 2012). The major GGPP synthase \textit{IdsA} (Heider et al., 2014a) and a second phytoene synthase are encoded elsewhere on the chromosome (Heider et al., 2012). Divergent to the \textit{crt} operon and 200 bp upstream of its first gene, \textit{crtE}, one of nine MarR-type transcriptional regulator genes in the genome of \textit{C. glutamicum} (Brune et al., 2005) is found. This gene \textit{cg0725} was named \textit{crtR} based on the results described in this study. Previously, a transposon mutagenesis screen showed that insertion of a transposon into \textit{crtR} enhanced carotenoid production (Krubasik et al., 2001a). A model of the protein secondary structure of the 195 aa containing \textit{crtR} product revealed a pafm12802 domain with a centrally located winged HTH motif located from aa position 73 to 134 as is typical for MarR-type regulator proteins of \textit{C. glutamicum} (Brune et al., 2005).

A sequence comparison by BLAST showed that \textit{C. glutamicum} CrtR is distinct from MarR-type regulators of \textit{Bacillus subtilis}, \textit{Escherichia coli}, \textit{Mycobacterium}, and \textit{Streptomyces} species (data not shown), but showed highest similarities to MarR-type proteins of the high GC content family of Actinobacteria, primarily of species of the genera \textit{Corynebacterium}, \textit{Microbacterium}, \textit{Leucobacter}, \textit{Arthrobacter}, \textit{Propionibacterium}, and \textit{Gulosibacter} (Figure S1). A sequence alignment with the closest relatives of CrtR (CAF19334) from \textit{C. vitaebuminis}, \textit{C. efficiens}, \textit{C. callunae}, \textit{Leucobacter} sp. Ag1, \textit{M. mangrovi}, \textit{Brevibacterium} sp. VCM10, and \textit{L. chironomi} shows high similarity especially in the central region of the proteins where the proposed HTH motif is located (Figure S2). Since \textit{crtR} is localized in proximity to the \textit{crt} operon in \textit{C. glutamicum}, it was tested if this pattern is conserved among bacteria containing a CrtR ortholog. First, sequence comparisons of \textit{C. glutamicum} CrtR to completely sequenced genomes identified bacteria possessing a CrtR homolog showing at least 25% amino acid identity and an e-value <10\textsuperscript{-10}. Subsequently, the completely sequenced genomes of these bacteria were analysed for the presence of homologs (25% amino acid identity and e-value <10\textsuperscript{-10}) of the \textit{C. glutamicum} proteins encoded by \textit{crtEb} (cg0717), \textit{crtYf} (cg0718), \textit{crtYe} (cg0719), \textit{crtI} (cg0720), \textit{crtB} (cg0721), \textit{crtE} (cg0723), \textit{crtX} (cg0730), and \textit{idsA} (cg2384). Although the low identity cut-off of 25% may have led to more false-positives than searches with higher identity cut-offs (Rost, 1999), this was compensated for by the high significance value of e\textsuperscript{-10} and the combinatorial search for CrtR homologs and other carotenogenesis genes. If at least one homolog of the proteins in this list were encoded in a completely sequenced genome in addition to a homolog of \textit{C. glutamicum} CrtR, the genome was considered further and the results depicted as heatmap (Figure S3). Opposite and in divergent orientation to the \textit{crtR} homologous genes, individual genes or \textit{crt} operons were found. For the most part, the individual genes or the first genes of the \textit{crt} operons encoded GGPP synthase (\textit{crtE}), IPP isomerase (\textit{idsi}), and putative multidrug efflux protein (\textit{mmpl}), respectively (Figure 1).

With respect to the genes encoding homologs of \textit{C. glutamicum} CrtR and genes involved in carotenogenesis
four distinct patterns emerged (Figure 1). First, a group of bacteria possessing a full complement of carotenogenesis genes including genes for carotenoid elongase (crtEb), carotenoid cyclase (crtY, crtY*/Y) and a carotenoid glycosyltransferase (crtX). This group comprised bacteria known to produce glycosylated, cyclic C50 carotenoids (C. glutamicum, C. callunae, C. marium, Micrococcus luteus), but also bacteria of which it is unknown which carotenoids they synthesize (Arthrobacter arilaitensis, Microbacterium Barkeri, and Microbacterium hydrocarbonoxydans; Figure 1). A second group with Agromyces subbeticus and Leucobacter chironomi lacks crtX homologs likely synthesizing non-glycosylated carotenoids and a third group with Propionibacterium jenseni and Corynebacterium lubricantis lacks crtX and crtY homologs likely synthesizing noncyclic nonglycosylated carotenoids. Finally, a group with Brevibacterium linens, Gulosibacter molinativorax and Timonella senegalensis lacks crtX, crtY and crtE/ubiA homologs and these are likely unable to synthesize glycosylated or cyclic or elongated carotenoids. Taken together, homologs of C. glutamicum CrtR are conserved in various bacteria (mostly actinobacteria) that were shown to synthesize various carotenoids (cyclic and noncyclic, elongated or not, glycosylated or not) or do have the potential to synthesize these according to their gene repertoire. Moreover, the crtR homologs are found in diverse genomic locations, but mostly adjacent to crt operons or genes involved in carotenogenesis. These findings supported the hypothesis that CrtR homologs may have a conserved role in regulation of carotenogenesis.

**CrtR Negatively Regulates Expression of the crt Operon and of Its Own Gene**

To test if *C. glutamicum* CrtR plays a role in transcriptional regulation of genes of carotenogenesis a *crt* deletion mutant was constructed and characterized with respect to global gene expression. Comparative transcriptome analyses of *C. glutamicum* MB001ΔcrtR and *C. glutamicum* MB001 were performed to identify genes directly or indirectly regulated by CrtR. The transcriptomes of cells cultured in triplicates either in glucose minimal medium or LB complex medium revealed only eight differentially expressed genes (Table 2). All of the differentially expressed genes showed 8 to 69-fold increased mRNA levels and they belonged to the *crt* operon or, as in the case of cg0726 that codes for a putative secreted lipoprotein, are localized adjacent to it. Deletion of *crtR* led to considerably higher mRNA ratios for cg0717 and cg0719 during growth in LB than in minimal medium, however, this does not pertain to the other genes of the *crt* operon. Currently, it remains unclear if these differences may be caused by technical issues or have a biological cause, however, it has to be noted that RNAseq analysis revealed expression of cg0717 and cg0718 independent of the biological cause, however, it has to be noted that RNAseq analysis revealed expression of cg0717 and cg0718 independent of the *crt* operon, thus, sub-operons may exist (Table 2). All of the differentially expressed genes showed 8 to 69-fold increased mRNA levels and they belonged to the *crt* operon or, as in the case of cg0726 that codes for a putative secreted lipoprotein, are localized adjacent to it. Deletion of *crtR* led to considerably higher mRNA ratios for cg0717 and cg0719 during growth in LB than in minimal medium, however, this does not pertain to the other genes of the *crt* operon. Currently, it remains unclear if these differences may be caused by technical issues or have a biological cause, however, it has to be noted that RNAseq analysis revealed expression of cg0717 and cg0718 independent of the other *crt* genes, thus, sub-operons may exist (Pfeifer-Sancar et al., 2013). As expected from the operon structure all transcripts were decreased from the first to the last genes in the *crt* operon. Taken together, the *crt* operon of *C. glutamicum* is directly or indirectly regulated by CrtR.

The pEPR1-promoter probe vector system for transcriptional fusion analysis (Knoppova et al., 2007) was used to determine...
if CrtR regulates transcription of its own gene and of the crt operon in vivo. The intergenic region between crtR and crtE, the first gene of the crt operon, was cloned upstream of the promoter-less gfp<sup>UV</sup> gene in both orientations (named p<sup>crtE</sup> and p<sup>crtR</sup>, respectively). Reporter gene expression in <i>C. glutamicum</i> MB001(pEPR1-P<sup>crtE</sup>), MB001(pEPR1-P<sup>crtR</sup>), MB001<sup>crtR</sup> (pEPR1-P<sup>crtR</sup>), and MB001<sup>crtR</sup> (pEPR1-P<sup>crtE</sup>) was determined by fluorescence cell scanning in LB medium in the early exponential growth phase (Table 3). About 2-fold higher expression of the P<sup>crtR</sup> fusion in the absence of CrtR revealed that CrtR is subject to negative autoregulation (Table 3). Expression of the P<sup>crtE</sup> fusion was about 5-fold higher when c<sup>rtr</sup> was deleted indicating that CrtR represses transcription of the <i>crt</i> operon (Table 3).

### Gel Mobility Shift and Transcriptional Fusion Analyses Revealed That CrtR Binds to the Intergenic Region between <i>crtR</i> and <i>crtE</i>

As CrtR was shown to negatively regulate transcription of the <i>crt</i> operon and of its own gene, gel mobility shift assays were performed to determine if CrtR directly interacts with the intergenic region between <i>crtR</i> and <i>crtE</i>. Therefore, CrtR protein was produced in recombinant <i>E. coli</i> BL21 (DE3) as N-terminal His-tagged protein and purified to apparent homogeneity by affinity chromatography (Figure S4). Gel mobility shift analysis revealed that His-tagged CrtR bound to the intergenic region between <i>crtR</i> and <i>crtE</i> (fragment A; Figures 2a,c), whereas BSA as a control did not bind to this DNA fragment (data not shown). At a 30 to 40-fold molar excess of CrtR a complete shift of the promoter fragment C was observed, whereas a negative control fragment comprising the sequence upstream of the unrelated gene cg2228 was not bound by CrtR (Figure 2b). To localize the CrtR binding site, truncated fragments of the intergenic region were used for further gel shift assays. In line with the observation that MarR-type regulators typically bind to palindromic sites of 16–20 bp which often overlap the −10 and −35 regions for steric inhibition of RNA polymerase (Wilkinson and Grove, 2006), two inverted repeats were found in the intergenic region between <i>crtR</i> and <i>crtE</i> (Figures 2a). CrtR bound to fragments A, C, D, E, and F that contained a TTA0A sequence motif 25–28 bases upstream of the transcription start site of <i>crtE</i> (Figures 2c,d). Fragments B and G that lack the complete TTA0A motif were not bound by CrtR (Figures 2c,d) indicating that this sequence motif might be essential for CrtR binding. Thus, this motif and a similar motif (AAATTT overlapping with the −10 hexamer of the <i>crtE</i> promoter; Figure 2a) were mutated. Mutating the latter motif (fragment H) did not influence binding of CrtR, whereas CrtR did not bind to fragments with a mutated TTA0A motif (fragments I and K; Figure 2d). Thus, the TTA0A motif was shown to be required for binding of CrtR in vitro.

In order to determine the role of the TTA0A motif for regulation by CrtR in vivo, transcriptional fusions of the promoters P<sup>crtR</sup> and P<sup>crtE</sup> containing mutations in the TTA0A and/or the AAATTT motifs were constructed. Mutation of the AAATTT motif changed the −10 hexamer of the P<sup>crtE</sup> promoter from TATAAA to TATTGG (Figure 2a). Consequently, only very low reporter gene activities were measured (Table 3) and thus,

### Table 2 | Genes differentially expressed in <i>C. glutamicum</i> strains MB001 and MB001<sup>crtR</sup> during growth in minimal medium with 100 mM glucose as well as in complex LB medium.

| Gene ID | Gene name | Annotation | LB | CgXII |
|--------|-----------|------------|----|-------|
| cg0717 | crtEb     | Lycopene elongase | 34 | 13    |
| cg0718 | crtYf     | C50 carotenoid epsilon cyclase | 33 | 35    |
| cg0719 | crtHe     | C50 carotenoid epsilon cyclase | 69 | 24    |
| cg0720 | cttl      | Phytoene dehydrogenase (desaturase) | 36 | 32    |
| cg0721 | cttR      | Phytoene synthase | 55 | 45    |
| cg0722 | –         | Putative multidrug efflux protein, resistance-nodulation-cell division (RND) superfamily | 13 | 22    |
| cg0723 | cttE      | GGPP synthase | 33 | 40    |
| cg0726 | –         | Putative secreted lipoprotein | 8  | 10    |

<sup>a</sup>Average ratio of medians. Only genes regulated more than 2-fold in at least one strain were considered. Only results with a <i>p</i>-value (adjusted with the False Discovery Rate approach) of ≤ 0.05 were considered to be significant. Three biological replicates were performed.

### Table 3 | Effect of <i>crtR</i> deletion on <i>crtE</i> and <i>crtR</i> promoter activity.

| pEPR1-LPCrtE | Native | M1* | M2* | M1*2* |
|-------------|--------|-----|-----|-------|
| MB001       | 0.21 ± 0.01 | 0.47 ± 0.02 | 3.89 ± 0.17 | 0.26 ± 0.01 | 0.79 ± 0.03 |
| MB001/crtR  | 0.20 ± 0.01 | 2.64 ± 0.09 | 4.12 ± 0.01 | 0.57 ± 0.01 | 0.82 ± 0.02 |
| pEPR1-LPCrtR | MB001 | 0.21 ± 0.01 | 0.63 ± 0.01 | 0.48 ± 0.01 | 0.63 ± 0.02 | 0.45 ± 0.01 |
| MB001/crtR  | 0.20 ± 0.01 | 1.10 ± 0.01 | 1.43 ± 0.01 | 0.44 ± 0.02 |

<i>GFP</i><sup>UV</sup> fluorescence medians of <i>C. glutamicum</i> MB001 strains with and without a genomic copy of <i>crtR</i> harboring either pEPR1-P<sup>crtR</sup> or pEPR1-P<sup>crtE</sup> or their mutated derivatives (M1*; motif 1 (TTAA → GCCC), M2*; motif 2 (AAATTT → CCCCGG)) as in Figure 2 when grown on LB complex. Cells were harvested in exponential growth phase. <i>GFP</i><sup>UV</sup> fluorescence was determined by FACS analysis. Mean values and standard deviations of three replicates are given.
the relevance of the AAATTT motif for regulation of the PcrtE promoter could not be determined. Reporter gene activities of a transcriptional fusion of PcrtR containing the mutated AAATTT motif were comparable to those obtained with the non-mutated PcrtR promoter and revealed that this motif is dispensable for negative autoregulation by CrtR (Table 3). Mutation of the TTAA motif led to very low reporter gene activity with PcrtR, which precluded deducing its involvement in crtr autoregulation. By contrast, mutation of the TTAA motif led to high reporter gene activities of PcrtE regardless of the absence or presence of CrtR (Table 3) which supports the notion that this motif is required for function of CrtR as transcriptional repressor of the crt operon in vivo.

Isoprenoid Pyrophosphates Prevent Binding of CrtR to Its DNA Target

Regulation of carotenogenesis in response to low-molecular-weight compounds has not yet been described. However, since CrtR belongs to the MarR family of transcriptional regulators and representatives of this family show regulation in response to low-molecular-weight compounds, such as hydrogen peroxide in the case of RosR of C. glutamicum (Bussmann et al., 2010), low-molecular-weight compounds were tested as effectors of CrtR in gel mobility shift assays. Intermediates of carotenogenesis (isoprenoid pyrophosphates), precursors of the MEP pathway (GAP and pyruvate), but also phosphorylated intermediates of glycolysis (DHA, phosphoenolpyruvate and D-glucosamine 6-phosphate), organic acids (acetate, propionate) and compounds affecting other MarR-type regulators [vitamin B12, salicylic acid, urea, protocatechuic acid ‘(data not shown)’] were tested as effectors of CrtR with concentrations in the mM range. Neither DHAP (Figure 3) nor pyruvate (data not shown) had an effect on CrtR binding to its DNA target. In the contrary, in the presence of GAP CrtR binding was prevented (Figure 3). In addition, the isoprenoid pyrophosphates IPP, DMAPP, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and GGPP perturbed binding of His-tagged CrtR to the intergenic region between crr and cte (Figure 3). Among these isoprenoid pyrophosphates, GGPP was effective at the lowest concentration (50 µM), whereas FPP, GPP, IPP and DMAPP exerted similar effects on CrtR binding in vitro at a 5-fold higher concentration.
Deletion of crtR Is a Metabolic Engineering Strategy to Increase Production of Native and Non-native Carotenoids

Since CrtR represses the crt operon the effect of deletion and overexpression of crtR on decaprenoxanthin accumulation in cells of C. glutamicum wild type and the phage-cured strain MB001 was determined. While growth was not significantly affected by the deletion or overexpression of crtR, neither in complex medium (data not shown) nor in minimal medium, the crtR deletion mutant showed more intense yellow pigmentation, while pigmentation of the overexpressing strain was slightly decreased (Figure S6). HPLC analyses of extracts from cells grown in CGXII medium revealed that the crtR deletion mutants accumulated decaprenoxanthin to 15 to 30-fold higher levels than the corresponding parental strains (Table 4). By contrast, as consequence of plasmid-borne overexpression of crtR decaprenoxanthin levels were significantly decreased (Table 4).

Since lycopene is a central intermediate of C40 and C50 carotenoid biosynthesis, the effect of deletion of crtR was elucidated in a metabolically engineered lycopene producing LYC5. Lycopene concentration increased about 5-fold (Figure 4). To test if this type of regulatory engineering can also be applied to the production of non-native carotenoids, crtR was deleted in the β-carotene producing strain BETA3, the sarcinaxanthin producing strain LYC5 (pEKEx3-crtE2Y), and the C.p.450 (2,2′-bis-(4-hydroxy-3-methybut-2-enyl)-β,β-carotene) producing strain LYC5 (pEKEx3-IbtABC). Indeed, all strains lacking CrtR produced 1.5 to 2 fold more carotenoid than the parental strains (Figure 4). Thus, deletion of crtR is a general strategy to boost production of native and non-native C40 and C50 carotenoids by C. glutamicum.

### DISCUSSION

In this study, the MarR-type transcriptional regulator CrtR was shown to repress the crt operon, its own gene and likely also the adjacent gene cg0726. GGPP and to lesser extent the isoprenoid pyrophosphates FPP, GPP, IPP, and DMAPP as well as the MEP pathway precursor GAP interfered with CrtR binding in vitro to a TTAA containing sequence in the intergenic region between crtR and the crt operon overlapping with the crt operon promoter.

CrtR is the first MarR-type regulator repressing carotenogenic genes in bacteria that is characterized to some detail. Commonly, RNA polymerase σ factors and transcriptional regulators of a different protein family (MerR-type, but not MarR-type) are involved in repression of carotenogenic operons in
is photolysed to hydroxocobalamin, which causes a large-scale conformational change of the regulator. This leads to the loss of operator binding and therefore derepression of its own gene and the subsequent cAMP-CRP family activator gene ldrP (Ortiz-Guerrero et al., 2011). This LdrP protein activates transcription of the crt operon, DNA photolyase gene phrB, and some further genes (Takano et al., 2014). LitR of S. coelicolor also functions as vitamin B12-dependent photoreceptor and its own gene and the ECF-σ factor gene lits are derepressed upon illumination. As consequence, RNA polymerase holoenzyme containing σ^{IIIS} transcribes the crt operons (Takano, 2016). B. megaterium possesses a LitR homologue as vitamin B12-dependent photoreceptor. The crt operon is relieved from repression by LitR in the light, however, a dedicated σ factor as in S. coelicolor is not involved since transcription occurs by RNA polymerase holoenzyme containing σ^{A} (Takano et al., 2015a). In C. glutamicum, however, the MarR-type transcriptional regulator CrtR has been shown here to repress the carotenoid biosynthesis operon. Based on the in vitro analysis it is hypothesized that isoprenoid pyrophosphates act as inducers. Hitherto, neither MarR-type regulators nor low-molecular-weight ligands, such as intermediates of the MEP-pathway or carotenogenesis have been reported to be involved in transcriptional regulation of microbial or plant carotenoid biosynthesis.

MarR-type transcriptional regulators are categorized according to their physiological function acting as regulators of (i) the stress response, (ii) virulence factors or (iii) aromatic catabolism (Wilkinson and Grove, 2006). Typically, MarR-type regulators are sensing environmental changes and some of them regulate drug efflux pump gene expression (Grove, 2013). C. glutamicum is equipped with nine MarR-type regulators (Brune et al., 2005) of which only the hydrogen peroxide-responsive activator of the nitrate/nitrite transporter and the dissipilatory nitrate reductase complex genes RosR (Bussmann et al., 2010), the repressor of the malic enzyme gene MalR (Krause et al., 2012), and PhdR, the repressor of the genes required for β-oxidation of phenylpropanoids (Alekhun et al., 2001), have been analyzed to date. MarR proteins are mostly encoded either as part of the regulated gene cluster or their genes are localized adjacent to and in divergent transcriptional organization to the regulated genes (Wilkinson and Grove, 2006). The latter case applies for CrtR, MalR, RosR, and PhdR of C. glutamicum WT. Although N-terminal HTH domains are most common for negative transcriptional regulators (Pérez-Rueda et al., 1998), CrtR possesses a central HTH domain preceded by 72 amino acids (corresponding to aa 73–134). This is also true for MalR, RosR and PhdR, however, their HTH domains are only preceded by 36 to 47 N-terminal amino acids and these proteins are about 40 amino acids shorter than CrtR.

The regulon of CrtR is small, comprising only the crt operon, its own gene and possibly cg0726. CrtR may have only one DNA binding site on the C. glutamicum genome which is located between crr and the crt operon and comprises a TTAA motif required for CrtR binding (Figure 2). This motif is located 25–28 bp upstream of the transcription start site of crr, which was identified 114 nucleotides upstream of the start codon (Heider et al., 2012). This motif overlaps the −35 and/or −10 promoter elements of the crt operon, which for other MarR-type regulators
was shown to indicate that repression is achieved by steric inhibition of RNA polymerase binding to the promoter (D’Souza et al., 1997). Although short, this motif is palindromic as generally observed for most MarR-type transcriptional regulators (Kelly et al., 1970; Ronnekleiv, 1995). When using promoter fusions of PcrtE and PcrtR, a more intense yellow pigmentation in comparison to the empty vector carrying control strains was observed (data not shown), which likely is due to a titrating effect by the CrtR binding site present on the medium copy promoter-probe vectors (Korshunov and Imlay, 2006). The more intense yellow pigmentation was not observed when the CrtR binding site in the promoter probe vectors was mutated. Deletion of crrmotif derepressed the crr operon by about 5-fold, but derepressed its own gene by about 2-fold, which might indicate that PcrtE is stronger than PcrtR. The −10 hexamer of PcrtE does not deviate from the consensus sequence TANNNT (Pfeifer-Sancar et al., 2013), while the −35 promoter hexamer TTTTAA consensus (TTGNCA) hexamer is less conserved. However, transcription of crrmotif and its autoregulation are less well understood since promoter motifs for crr cannot easily be found (Figure 2a), although it has been reported that crr is transcribed as a leaderless transcript (Pfeifer-Sancar et al., 2013). A surprisingly high fraction of transcripts in C. glutamicum (33%) lack an 5′ untranslated region (Pfeifer-Sancar et al., 2013) and it remains to be studied if leaderless transcripts are translated less efficient than transcripts containing leaders (Moll et al., 2004) and if they are resistant to inhibition by kasugamycin as is the case for E. coli (Lange et al., 2016).

MarR-type regulators are often involved in stress responses and conformational changes due to binding of an effector molecule impair their binding to their specific DNA targets, as it was described e.g., for MarR in its salicylated form (Alekhun et al., 2001). The MarR-type regulator of oxidative stress response RosR of C. glutamicum showed reduced binding to its target DNA when exposed to H2O2. Furthermore, H2O2 sensitivity was increased in the absence of the rosR adjacent gene cg1322, which is proposed to encode a secreted protein likely binding an octaprenyl pyrophosphate molecule. Since menaquinone contains an octaprenyl side chain and has an effect on the flux of superoxide to the periplasm of E. coli (Korshunov and Imlay, 2006), a correlation between oxidative stress and polyisoprenoid structures in the cytoplasmic membrane of C. glutamicum has been postulated (Bussmann et al., 2010). In this study, an influence of typical MarR effectors, such as acetate, propionate, salicylic acid, urea or protocatechueic acid could not be observed (data not shown). Vitamin B12 involved in LitR-mediated control of carotenogenesis in S. coelicolor (Takekano et al., 2005), did not affect binding of CrtR to PcrtE. However, we cannot exclude that the CrtR preparations from E. coli are free from low molecular weight compounds.

Here, we have described that isoprenoid pyrophosphates interfered with binding of CrtR to its cognate DNA target. Consistently, many members of the MarR family bind similar, mostly anionic lipophilic or even phenolic effector molecules (Wilkinson and Grove, 2005). The interfering effect of isoprenoid pyrophosphates was specific as vitamin B12, phosphoenolpyruvate, D-glucosamine 6-phosphate, acetate, propionate, salicylic acid, urea or protocatechueic acid had no significant influence on CrtR binding (data not shown). Based on these in vitro results, it is tempting to speculate that CrtR may be used as a potential biosensor of IPP, DMAPP, GPP, FPP, and GGPP for strain engineering and optimizing terpenoid production by controlling gene expression on demand. This kind of strain development has been shown to work efficiently using photocaged-IPTG as applied to valencene production by C. glutamicum (Binder et al., 2016) or using riboswitches as applied to lysine production by C. glutamicum (Zhou and Zeng, 2015a,b).

CrtR is not only found in corynebacteria, but conserved in actinobacteria that, based on their genome content, have the potential to synthesize various carotenoids (Figure 1). It has to be noted that the genomic organization of crr in most of these bacteria is linked to genes involved in carotenogenesis. Thus, it is not unlikely that CrtR plays a comparable role in transcriptional regulation of carotenogenic genes in these bacteria.

AUTHOR CONTRIBUTIONS

All authors planned and designed the experiments. NAH, SAEH, and SH performed the experiments and analyzed the data. PPW and VFW analyzed the data. NAH, SAEH, and PPW drafted the manuscript. VFW and PPW coordinated the study and finalized the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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