ARTICLE

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# Characterization of Cyanobacterial β-Carotene Ketolase and Hydroxylase Genes in *Escherichia coli*, and Their Application for Astaxanthin Biosynthesis

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**ABSTRACT:** Carotenoid biosynthesis is highly conserved and well characterized up to the synthesis of β-carotene. Conversely, the synthesis of astaxanthin from β-carotene is less well characterized. Regardless, astaxanthin is a highly sought natural product, due to its various industrial applications and elevated antioxidant capacity. In this article, 12 β-carotene ketolase and 4 β-carotene hydroxylase genes, isolated from 5 cyanobacterial species, are investigated for their function, and potential for microbial astaxanthin synthesis. Further, this in vivo comparison identifies and applies the most promising genetic elements within a dual expression vector, which is maintained in *Escherichia coli*. Here, combined overexpression of individual β-carotene ketolase and β-carotene hydroxylase genes, within a β-carotene accumulating host, enables a 23.5-fold improvement in total carotenoid yield (1.99 $mg\,g^{-1}$), over the parental strain, with >90% astaxanthin.

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**KEYWORDS:** *E. coli*; astaxanthin; cyanobacteria; metabolic engineering; β-carotene ketolase; β-carotene hydroxylase

## Introduction

Natural products comprise a vast group of biologically diverse and important compounds. Carotenoids, one such class of natural products, are represented by over 600 novel structures (Del Campo et al., 2007; Krinsky and Johnson, 2005). In nature, carotenoids are abundant in number, but present in trace amounts. This presents a significant limitation on carotenoid research, and their potential industrial, nutraceutical, and pharmaceutical applications. Astaxanthin, a rare di-hydroxyl di-keto carotenoid, is a potent antioxidant, with potential applications in the treatment and/or prevention of a number of diseases (Guerin et al., 2003; Hussein et al., 2006; Krinsky and Johnson, 2005). Astaxanthin is also employed widely as a component of the feed used by fisheries and poultry farms (Aflalo et al., 2007; Cifuentes et al., 2003; Lorenz and Cysewski, 2000). Thus, in 2006, astaxanthin had an estimated commercial value of US $206 million (Global Industry Analysts, 2006).

Much research has focused on astaxanthin production. However, success has been limited, with natural synthesis schemes being restricted by the physiology of native producers, and the resulting un-economical fermentation strategies, leading to relatively low final product yields (Aflalo et al., 2007; Ausich, 1997; Del Campo et al., 2007; Rio et al., 2007; Tinoi et al., 2006), this is in spite of over 25 years of research in the field (Borowitzka et al., 1991; Johnson et al., 1978). Chemical synthesis provides an alternative to biological production, but is also far from ideal, being restricted by the complex structure of astaxanthin, and issues regarding non-specific stereoisomer production (Choi and Koo, 2005; Coral-Hinostroza et al., 2004). To date, metabolic engineering studies and heterologous production strategies have failed to provide a method by which commercial demand can be satisfied.

Carotenoid biosynthesis proceeds in a highly conserved, linear manner from the $C_5$ precursors isopentenyl

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pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP), through to β-carotene (Fig. 1). From β-carotene, synthesis may proceed via a number of routes, culminating with the synthesis of astaxanthin (Fig. 1). In the synthesis of astaxanthin, from β-carotene, the catalytic activity of two classes of protein is required. The β-carotene ketolase class of proteins, encompassed by CrtO and CrtW types, catalyze the addition of a ketone group to carbon 4/4′ of the β-ionone ring, while β-carotene hydroxylase, represented by CrtZ, CrtR, and P450 types, catalyze the hydroxylation of carbon 3/3′ of the β-ionone ring. The activity of these proteins, along with the characterization of the steps involved in the synthesis of astaxanthin from β-carotene has been the focus of much research. The reader is directed to the literature for a deeper discussion on this topic (Choi et al., 2007; Lee and Schmidt-Dannert, 2002; Lu and Li, 2008). The focus of this article is to elucidate an improved combination of genes, for the microbial production of astaxanthin. Cyanobacteria are recognized as prolific producers of secondary metabolites (Burja et al., 2001), and like all photosynthetic organisms are ubiquitous producers of carotenoid compounds. Here, we present the characterization of 12 cyanobacterial-derived β-carotene ketolase and 4 β-carotene hydroxylase genes, from 5 different cyanobacterial species, within *Escherichia coli*. *E. coli* was selected as the host system due to its prosperity in the biosynthesis of carotenoid compounds (Das et al., 2007), and in the commercial production of other bioproducts (Lee, 1996; Shiloach and Fass, 2005). These carotenoid biosynthetic genes are compared in vivo, to allow for selection and combination of the most appropriate elements. Intrinsic to this is the development of a dual expression vector system, via the application of a novel PCR mediated cloning method, also described here, which enables rapid cloning of target genes into any predetermined expression vector.

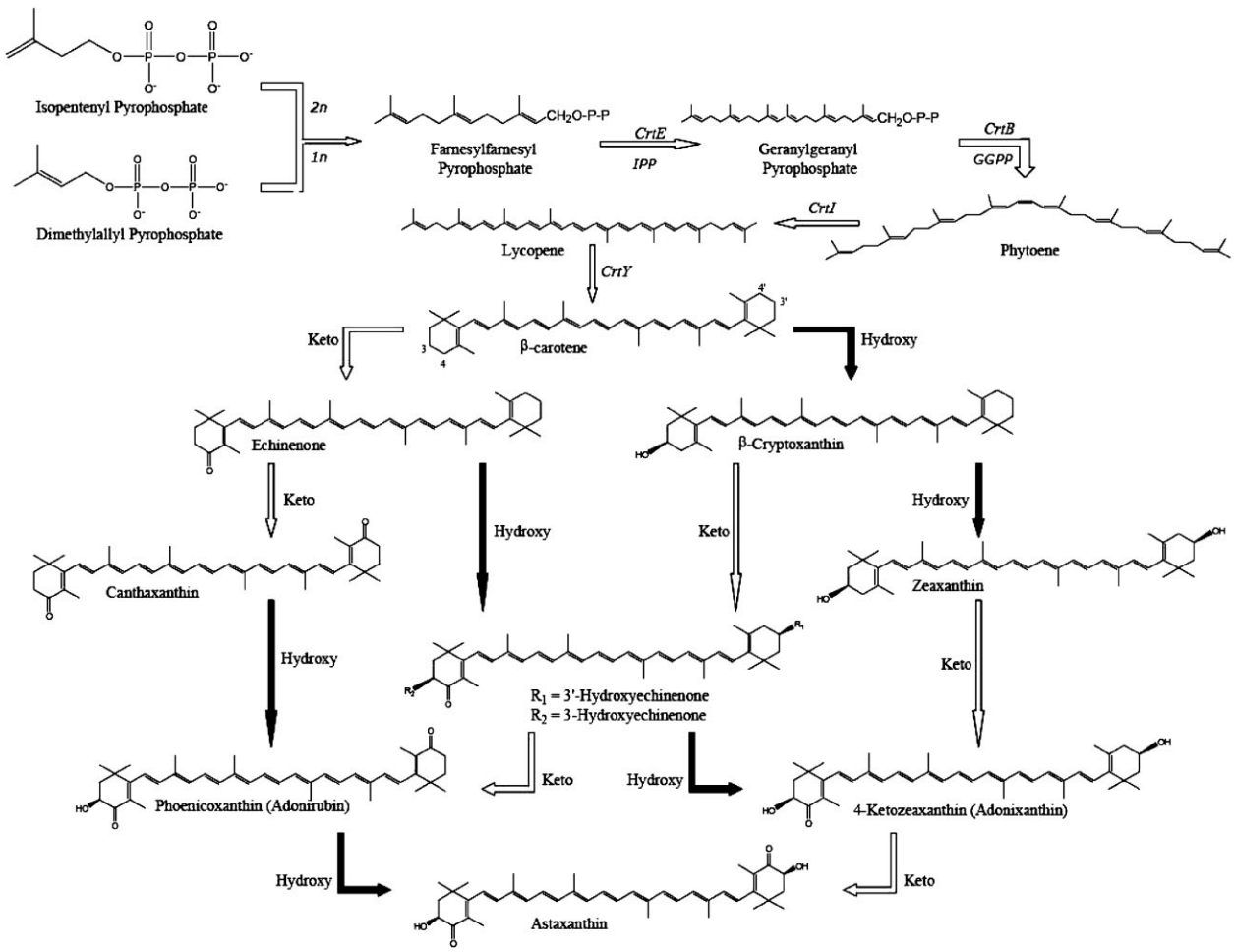


**Figure 1.** Astaxanthin biosynthetic pathway, proceeding from isopentenyl diphosphate and dimethylallyl diphosphate, highlighting the numerous routes by which synthesis may proceed, and the relative (predicted) roles of β-carotene ketolase (Keto) and β-carotene hydroxylase (Hydroxy) proteins.

## Materials and Methods

### Bioinformatic Screen of Cyanobacterial Genomes

GenomeBLAST (Altschul et al., 1997) was used to screen the translated genomes (downloaded from NCBI, July 17th 2006) of cyanobacterial strains *Synechocystis* sp. PCC 6803, *Anabaena variabilis* ATCC 29413, *Nostoc punctiforme* PCC 73102, *Nostoc* sp. PCC 7120, and *Gloeobacter violaceus* PCC 7421. The β-carotene ketolase, CrtO type from *Synechocystis* sp. PCC 6803 (Fernandez-Gonzalez et al., 1997), CrtW type from *N. punctiforme* PCC 73102 (Steiger and Sandmann, 2004), and β-carotene hydroxylase, CrtR type from *Synechocystis* sp. PCC 6803 (Masamoto et al., 1998), amino acid sequences were employed as the query. Genes to be investigated were selected based on a statistical cut off (*E*-value) of $10^{-50}$.

### Bacterial and Cyanobacterial Strains and Culture Conditions

Cyanobacterial strains were purchased from the Culture Collection of Algae and Protozoa (CCAP, Argyll, UK) (www.ccap.ac.uk), cultured in a plant growth chamber (Sanyo, Osaka, Japan), at 25°C, 50 $\mu E\,m^{-2}\,sec^{-1}$ illumination, 60% humidity, on a 12 h light/dark regime, in BG11 media (Stanier et al., 1971). *E. coli* strain TOP10 (Invitrogen, Paisley, UK) was used for all cloning and expression work. *E. coli* was cultured in Luria–Bertani broth (Miller) (Sambrook et al., 1989), at 37°C, 150 rpm. Heterologous protein expression and carotenoid biosynthesis was performed at 30°C, 150 rpm, in the dark. Ampicillin and chloramphenicol were used at 50 $\mu g\,mL^{-1}$, as required. Unless otherwise stated, heterologous gene expression was induced with 2% L-arabinose.

### DNA Extraction and Purification

Cyanobacterial genomic DNA was extracted as previously described (Tamagnini et al., 1997). Following extraction, genomic DNA was diluted to 100 ng $\mu L^{-1}$ with nuclease free water and further purified via the addition of 900 μL of solution MD3 (MoBio Ultraclean microbial DNA isolation kit, MoBio, Carlsbad, CA) to 20,000 ng genomic DNA. The solution was mixed by vortexing for 5 s, loaded onto a single spin filter, and centrifuged at 16,500*g* for 30 s. DNA was washed with 300 μL Solution MD4 (MoBio), via centrifugation at 16,500*g* for 30 s, and eluted into a sterile 1.7 mL eppendorff tube with 50 μL of Solution MD5 (MoBio). Eluted genomic DNA was diluted to 100 ng $\mu L^{-1}$ with nuclease free water.

### Gene Amplification and Plasmid Construction

Identified, putative genes were isolated and amplified via PCR, using oligo-nucleotide primers, designed in-house, and synthesized by Operon Biotechnologies GmbH (Table I). PCR was performed on 1 μL genomic DNA using *Taq* DNA polymerase (Sigma, Dorset, UK), as per the manufacturer's instructions. PCR products were purified prior to cloning, via the application of SureClean (Bioline, London, UK), as per the manufacturer's instructions. Following resuspension in 20 μL nuclease free water, 4 μL PCR product was immediately utilized in a cloning reaction with plasmid pTrcHis2-TOPO (Invitrogen), and transformed into competent *E. coli* TOP10 cells, according to the manufacturer's instructions. Positive colonies were identified via colony screen PCR and sequence comparison to GenBank entries to confirm plasmid integrity and target gene fidelity.

### PCR Mediated Cloning

Target genes to be functionally expressed within plasmid pBAD24 (NCBI Accession No: X81837) (Guzman et al., 1995), were amplified via PCR, using oligo-nucleotide primers detailed in Table II. Briefly, to 50 ng template DNA the following was added; 0.25 μL Bio-X-ACT Short DNA polymerase (4 U $\mu L^{-1}$) (Bioline), 2.5 μL 10 × Opti buffer, 1.5 μL magnesium chloride (50 mM), 0.5 μL 10 mM dNTP mix (Promega, Southampton, UK), 1 μL (10 pmol) of each primer, forward and reverse (Table II), nuclease free water to 25 μL (Promega). PCR was performed as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 60 s, 49°C for 30 s, and 68°C for 60 s, followed by a single incubation at 68°C for 10 min. Plasmid pBAD24 was prepared via cleavage with *Nco*I (unique restriction site at residue 1318 (C/CATGG)). The PCR mediated cloning reaction was as follows: 1 μL PCR product, 1 μL *Nco*I digested pBAD24, were added to the following; 0.5 μL Bio-X-ACT Long DNA polymerase (4 U $\mu L^{-1}$) (Bioline), 2.5 μL 10× Opti buffer, 1.5 μL $MgCl_2$ (50 mM), 0.5 μL 10 mM dNTP mix (Promega), with nuclease free water added to final volume of 25 μL (Promega). PCR was performed with the parameters set at 94°C for 4 min, followed by 30 cycles of 94°C for 60 s, 60°C for 30 s, and 68°C for 5 min, with a single incubation at 68°C for 10 min. Subsequently, 5.5 μL NEB 4 buffer and 1 μL *Dpn*I (20 U $\mu L^{-1}$) (New England Biolabs, Hitchin, UK) were added to the PCR mixture, followed by incubation at 37°C for 2 h, and inactivation of the endonuclease at 80°C for 20 min. Two microliters of this solution was then used to transform TOP10 *E. coli* cells (Invitrogen). Transformation and outgrowth were performed according to the manufacturer's instructions. Positive colonies were identified via PCR screen.

### PCR Mediated Cloning: Creation of Dual Expression Vectors

In the creation of this series of dual expression vectors, all DNA regions required for efficient target gene expression (*AraC*, pBAD promoter, and target gene), within an *E. coli*

**Table I.** Oligo-nucleotide primers employed for amplification of cyanobacterial β-carotene ketolase and hydroxylase genes.

| Target ORF | Organism | Primer name | Sequence (5–3′) | $T_m$ (°C) |
|---|---|---|---|---|
| ZP_00111616 | *Nostoc punctiforme* PCC 73102 | Np.1 CrtO Ftrc | ATGCAAGAGTATGATGTTGTGCTGATCG | 63 |
| | | Np.1 CrtO R | CGAATCTCTTGCATCCTTTAAGGTTTGGCTAATAGGATG | |
| ZP_00112486 | *Nostoc punctiforme* PCC 73102 | Np.2 CrtO Ftrc | ATGGAATCGTATGACGTTGTAATTATTGGCGCTGGTC | 64 |
| | | Np.2 CrtO R | CAGCCTCTTAGTCTGATTCACTCCTCAAGACAGATG | |
| YP_322099 | *Anabaena variabilis* ATCC 29413 | Av CrtO Ftrc | ATGCAAGAGTATGATGTTGTAATGATTGGTGC | 63 |
| | | Av CrtO R | GTCATCAGTAGTCAACCGTCAACCGTCAAC | |
| NP_487784 | *Nostoc* sp. PCC 7120 | Asp CrtO Ftrc | ATGCAAGAATATGATGTTGTAATGATTGGTGCAG | 63 |
| | | Asp CrtO R | GTCGTCAGTAGTCAACCGTCAACCGTTAACTGTCAAC | |
| NP_923340 | *Gloeobacter violaceus* PCC 7421 | Gv CrtO Ftrc | ATGCCAGGGAGTTCGGACGTGG | 56 |
| | | Gv CrtO R | CGACCAGCTGCCGGTCAGGCTGCGGTACACC | |
| NP_442491 | *Synechocystis* sp. PCC 6803 | Syn pTrcF | ATGATCACCACCGATGTTGTC | 56 |
| | | Syn pTrcR | GCTGTACCAAAAACGACGTTG | |
| YP_322565.1 | *Anabaena variabilis* ATCC 29413 | pTrcAv.1WF | ATGACTGAATGTAATTCCATATCTTTGC | 55 |
| | | pTrcAv.1WR | GTAATTATTATTATAAACATCTGGTAATCTCC | |
| ZP_00345866.1 | *Nostoc punctiforme* PCC 73102.1 | pTrc73102.1WF | ATGATCCAGTTAGAACAACCACTCAG | 56 |
| | | pTrc73102.1WR | GTATTTTGCTTTGTAAATTTCTGGTAACTGC | |
| YP_324388.1 | *Anabaena variabilis* ATCC 29413 | pTrcAv.2WF | ATGGTTCAGTGTCAACCATCATCTCTACG | 55 |
| | | pTrcAv.2WR | GTATAAAGATATTTTGTAAGCTTCCGG | |
| NP_487229.1 | *Nostoc* sp. PCC 7120 | pTrc7120WF | ATGGTTCAGTGTCAACCATCATCTCTGC | 55 |
| | | pTrc7120WR | GTATAAAGATATTTTGTGAGCTTCAGG | |
| ZP_00111258.2 | *Nostoc punctiforme* PCC 73102.2 | pTrc73102.2WF | ATGAATTTTTGTGATAAACCAGTTAGCTATTATGTTGC | 56 |
| | | pTrc73102.2WR | GTACGAATTGGTTACTGAATTGTTGAATAC | |
| NP_924674.1 | *Gloeobacter violaceus* PCC 7421 | pTrc7421WF | ATGATGCGTGGCTCGGCAGTAAAGGAACG | 65 |
| | | pTrc7421WR | GTAGCGGCGCGCCTCGGGCAGCCGATGC | |
| ZP_00106832.1 | *Nostoc punctiforme* PCC 73102 | Np CrtR F | ATGTTAGAGATGATCGCATCGG | 55 |
| | | Np CrtR R | ATATTCTTTTTGATGATGAAACCGAATTCC | |
| YP_322210.1 | *Anabaena variabilis* ATCC 29413 | Av CrtR F | ATGCTCTCATTGGAGGC | 51 |
| | | Av CrtR R | ATATGCCTCTAGATTATTACTTG | |
| NP_488049.1 | *Nostoc* sp. PCC 7120 | Nsp CrtR F | ATGCTTTTATTTTCGGCATCGGTGGTC | 54 |
| | | Nsp CrtR R | ATATGCCTCTAGATTATTACTTGATAC | |
| NP_440788.1 | *Synechocystis* sp. PCC 6803 | Syn CrtR F | ATGCAGGCGACCCAACCG | 55 |
| | | Syn CrtR R | ATAGGGCTTGTCAGATGATTGAG | |
| M87280 | *Erwinia herbicola* | Eherb CrtZ F | ATGCTAGTAAATAGTTTAATCGTCATC | 55 |
| | | Eherb CrtZ R | TTATTCGGGCGAAGACGACGAGG | |

host, were isolated and amplified, via PCR. Furthermore, these modified pBAD24 plasmids, the creation of which is described above, were employed as the template DNA (Table IV) for dual expression vector creation. This reaction consisted of 100 ng template DNA, 1 μL Bio-X-ACT Long DNA polymerase (4 U $\mu L^{-1}$) (Bioline), 5 μL 10× Opti buffer, 3 μL $MgCl_2$ (50 mM), 1 μL dNTP mix (10 mM) (Promega), 2 μL each primer (Table III), with nuclease free water added to constitute a final volume of 50 μL (Promega). PCR was performed with the parameters: 94°C for 4 min, and 30 cycles of 94°C for 60 s, 50°C for 30 s, and 68°C for 2 min. This was followed by a single incubation at 68°C for 10 min.

Template DNA (modified pTrcHis2 plasmid), for insertion of the pBAD expression cassette was prepared via *Sal*I digestion (5′-G/TCGAC-3′ unique cleavage site). To

**Table II.** Oligo-nucleotide primers used for amplification and linker attachment to CrtW type β-carotene ketolase genes.

| ORF ID | Primer name | Primer sequence (5–3′) |
|---|---|---|
| ZP_00345866.1[a] | 73102.1WF-LINKER | GGCTAGCAGGAGGAATTCACCATGATCCAGTTAGAACAACCAC |
| | 73102.1WR-LINKER | CGACTCTAGAGGATCCCCGGGTACGTATTTTGCTTTGTAAATTTCTG |
| NP_487229.1[a] | 7120WF-LINKER | GGCTAGCAGGAGGAATTCACCATGGTTCAGTGTCAACCATC |
| | 7120WR-LINKER | CGACTCTAGAGGATCCCCGGGTACGTATAAAGATATTTTGTGAGC |
| YP_324388.1[a] | Av.2WF-LINKER | GGCTAGCAGGAGGAATTCACCATGGTTCAGTGTCAACCATCATC |
| | Av.2WR-LINKER | CGACTCTAGAGGATCCCCGGGTACGTATAAAGATATTTTGTAAGCTTC |
| NP_442491[a] | SynOF-LINKER | GGCTAGCAGGAGGAATTCACCATGATCACCACCGATGTTGTC |
| | SynOR-LINKER | CGACTCTAGAGGATCCCCGGGTACTTACCAAAAACGACGTTG |
| M87280[a] | EherbZF-LINKER | GGCTAGCAGGAGGAATTCACCATGCTAGTAAATAGTTTAATCGTC |
| | EherbZR-LINKER | CGACTCTAGAGGATCCCCGGGTACTTATTCGGGCGAAGACGACGAGG |

Underlined is the linker sequence.
[a]See Table I for organism information.

**Table III.** Oligo-nucleotide linker primer details for amplification and extension of pBAD24 expression cassette in preparation for insertion into target vectors.

| Primer | Sequence |
|---|---|
| pBAD to pTrc F | TACGTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGCCGTCAATTGTCTGATTCGTTACC |
| pBAD to pTrc R | CAGCCAAGCTGGAGACCGTTTAAACTCAATGATGATGATGATGCCTGCAGGTCGACTCTAGAGGATC |

achieve this, 2 μg of plasmid DNA was combined with 3 μL of NEB 3 buffer, 0.5 μL Bovine Serum Albumin (BSA) (10 $\mu g\,\mu L^{-1}$) and 2 μL *Sal*I (20 $U\,\mu L^{-1}$) (New England Biolabs), nuclease free water (Promega) was added to 30 μL final volume. Digestion was performed at 37°C for 4 h. Target plasmids, and expression cassette combinations for dual expression vector construction are detailed in Table IV. PCR products and *Sal*I digested target plasmid were purified using SureClean (Bioline), according to the manufacturer's instructions, and eluted in 20 μL nuclease free water prior to application in the PCR mediated creation of novel dual expression vectors. Briefly this was composed of: 100 ng amplified pBAD expression cassette, 1 μL *Sal*I digested target plasmid DNA, 5 μL 10× Opti buffer, 2 μL 50 mM magnesium chloride, 1 μL 10 mM dNTP mix, 1 μL Bio-X-ACT DNA polymerase (4 $U\,\mu L^{-1}$) (Bioline), and nuclease free water to 50 μL (Promega). PCR was performed with the parameters set at 94°C for 4 min, followed by 30 cycles of 94°C for 60 s, 60°C for 30 s, and 68°C for 5.5 min, followed by a single incubation at 68°C for 10 min. Amplification reactions were subsequently digested with *Dpn*I, via the addition of 5.5 μL NEB 4 buffer and 1 μL *Dpn*I, to the reaction mixture. Incubation, inactivation and transformation of *E. coli* strain TOP10, were performed as previously described.

## Carotenoid Biosynthesis

Functional characterization of target genes was achieved via co-expression, in *E. coli* TOP10 cells, with either pAC-BETA or pAC-ZEAX (Cunningham et al., 1996; Sun et al., 1996). In this, three colonies were selected and each expressed in duplicate, for each target gene. Expression was performed in 100 mL cultures, as described previously. For pTrc expression systems, the addition of 1 mM IPTG was found to be detrimental to carotenoid biosynthesis, thus basal expression was exploited. Following incubation at 30°C, 150 rpm, in the dark, for 24 h, cells were harvested via centrifugation at 4°C and lyophilized (Edwards, Freeze Dryer Modulyo (Northern Scientific, York, UK), Edwards, Two Stage High vacuum pump), the biomass weighed to 4-decimal places (Fisherbrand, Loughborough, UK, PC-200) and stored at −80°C until extraction and analysis was performed.

**Table IV.** Combinations employed in creation of six dual expression vectors.[a]

| Target plasmid | Expression cassette to be inserted |
|---|---|
| pTrc-CrtZ Eherb | pBAD-CrtW Av 29413 (.2)<br>pBAD-CrtW Nostoc 7120<br>pBAD-CrtW Np 73102 (.1) |
| pTrc-CrtW Av 29413(.2)<br>pTrc-CrtW Nostoc 7120<br>pTrc-CrtW Np 73102(.1) | pBAD-CrtZ Eherb |

[a]See Table I for target ORF and organism information.

## Carotenoid Extraction

To the lyophilized biomass, in a darkened room, 5 mL of ice-cold methanol (HPLC grade, Fisher Scientific, Loughborough, UK) was added. Biomass was then ground with a mortar and pestle to a visually homogenous solution, and vortexed at high speed for 30 s. Extraction was performed on ice for 20 min, with shaking at 150 rpm, followed by vortexing at high speed for 30 s. Cell debris was removed via centrifugation at 3,500*g* for 20 min, at 4°C. The methanol extract was transferred to a chilled amber glass 12 mL screw top vial, and dried under a stream of nitrogen. The extract was then resuspended 250 μL ice-cold methanol, transferred to a chilled 1.7 mL eppendorff tube, and residual cell debris removed prior to HPLC analysis, by centrifugation at 16,500*g*, at 4°C, for 5 min. Samples were stored on ice, in the dark until HPLC analysis. All extracted samples used for identification and quantification were analyzed via HPLC within 4 h of extraction, if not these were stored dry at −80°C and analyzed the following day in order to minimize degradation.

## HPLC Analysis

Carotenoid analysis was performed as described previously (Armenta et al., 2006). Briefly this analysis employed an Agilent 1100 HPLC system equipped with a Phenomenex, Luna C18, 4.6 mm × 250 mm, 5 μm column, in conjunction with a Phenomenex SecurityGuard C18, 4 μm × 3.0 μm guard column. Detection was performed at 474 nm. The mobile phase, and gradient used was as follows: 100% buffer A (ethyl acetate/methanol/water (10:88:2)) was isocratic at 0.75 $mL\,min^{-1}$ for 10 min, increasing to 100% buffer B (ethyl acetate/methanol/water (48:50:2)), and 1.5 $mL\,min^{-1}$ over 20 min. Standard curves were generated using commercial standards: β-carotene (Sigma), astaxanthin (Sigma), canthaxanthin (CaroteNature, Lupsingen, Switzerland), and zeaxanthin (CaroteNature). Additional carotenoids, β-cryptoxanthin, echinenone, and lycopene, were identified via retention time, by comparison to a complex carotenoid standard provided by Dr. Adam Burja (Ocean

Nutrition Canada Ltd, Dartmouth, Nova Scotia) (supplementary material 3). Quantification of echinenone was approximated via application of the canthaxanthin standard curve.

## Results

### Functional Expression of Cyanobacterial CrtO β-Carotene Ketolase Genes, in β-Carotene Accumulating *E. coli*

In silico analysis of five cyanobacterial genomes elucidated 12 putative β-carotene ketolase genes, 6 CrtO, and 6 CrtW. ClustalW analysis revealed significant diversity within each ketolase class (Refer to supplementary material 1 and 2). Although all CrtO ketolases did encode six conserved motifs, previously identified as indicative of a functional CrtO ketolase (Tao and Cheng, 2002), while all CrtW ketolases encoded three highly conserved histidine rich motifs, conserved both in the primary and tertiary sequence of the protein. These are thought to be characteristic of a functional CrtW ketolase (Fraser et al., 1997; Ye et al., 2006).

Each putative ketolase gene (refer to Tables I and II for GenBank accession numbers) was expressed in *E. coli*, harboring plasmid pAC-BETA. Such *E. coli* strains produced β-carotene at 85 $\mu g\,g^{-1}$ (164 $\mu g\,L^{-1}$). Co-expression demonstrated that all identified putative CrtO β-carotene ketolase genes did encode functional proteins, evidenced by the synthesis of keto-carotenoids (Fig. 2A). In this work, all experimental variables were fixed, enabling a direct in vivo comparison of catalytic activity. This revealed that catalytic activity varied significantly within the CrtO class of protein, while also identifying the CrtO ketolase, isolated from *Synechocystis* sp. PCC 6803, as the most active within this sample group (Fig. 2A). Incidentally, this protein was also the only CrtO type ketolase able to catalyze the synthesis of both the mono- and di-keto carotenoids, echinenone, and canthaxanthin, a property previously alluded to (Lee et al., 2003). This work therefore confirms the previous characterization of CrtO β-carotene ketolase genes isolated from *Synechocystis* sp. PCC 6803 (Fernandez-Gonzalez et al., 1997; Lee et al., 2003) and *G. violaceus* PCC 7421 (Steiger et al., 2005; Tsuchiya et al., 2005), while also representing the first functional characterization of three additional CrtO β-carotene ketolase genes. Of these, two were isolated from *N. punctiforme* PCC 73102, and one was isolated from *A. variabilis* ATCC 29413. Furthermore, this work represents the first functional characterization, via heterologous

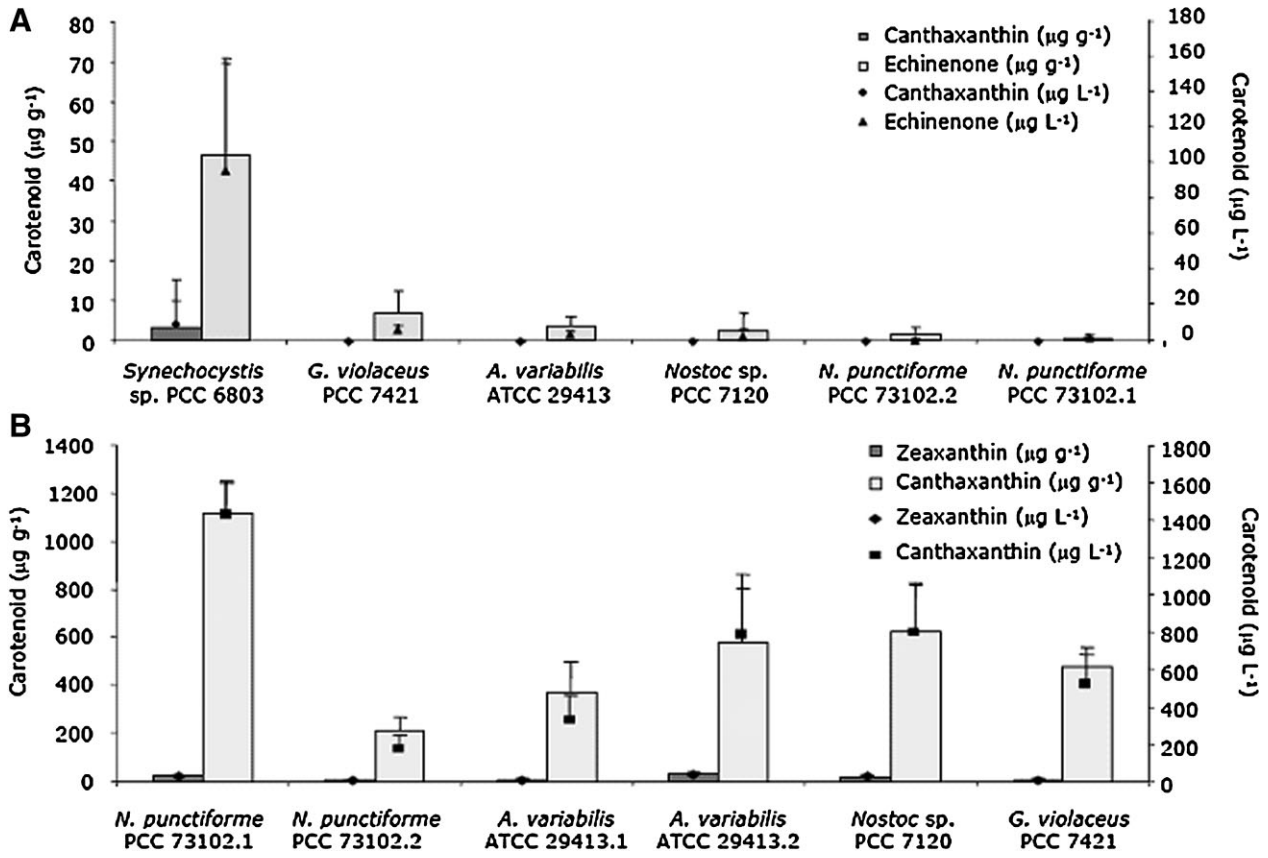


**Figure 2.** Keto-carotenoid biosynthesis, due to heterologous expression of cyanobacterial (**A**) CrtO and (**B**) CrtW β-carotene ketolase genes, within β-carotene accumulating TOP10 *E. coli*. Values represent the mean ($n \geq 3$), while error bars represent the standard deviation.

expression, of the previously identified CrtO β-carotene ketolase derived from *Nostoc* sp. PCC 7120 (Mochimaru et al., 2005).

Heterologous expression of each putative CrtW β-carotene ketolase protein, within β-carotene biosynthesizing *E. coli*, confirmed the functionality of a further six putative proteins. Thus, this work also confirmed the function of previously characterized CrtW ketolases identified from *N. punctiforme* PCC 73102 (Steiger and Sandmann, 2004) and *G. violaceus* PCC 7421 (Steiger et al., 2005; Tsuchiya et al., 2005). Additionally this work represents the first functional characterization, via heterologous expression, of the previously identified CrtW ketolase of *Nostoc* sp. PCC 7120 (Mochimaru et al., 2005), and the first functional characterization of two novel CrtW ketolase genes isolated from *A. variabilis* ATCC 29413. The in vivo comparison revealed that of the CrtW ketolases investigated, three demonstrate enhanced catalytic activity, as determined by keto carotenoid yield. These were isolated from *N. punctiforme* PCC 73102, *A. variabilis* ATCC 29413, and *Nostoc* sp. PCC 7120 (NCBI accession numbers: ZP_00345866.1, YP_324388.1, and NP_487229.1), catalyzing the synthesis of 1,113.8 $\mu g\,g^{-1}$ (1,430 $\mu g\,L^{-1}$), 578.8 $\mu g\,g^{-1}$ (783.2 $\mu g\,L^{-1}$), and 621.6 $\mu g\,g^{-1}$ (804 $\mu g\,L^{-1}$) canthaxanthin, respectively (Fig. 2B). In all instances, heterologous expression of the cyanobacterial CrtW ketolase genes, within β-carotene producing *E. coli*, resulted in the predominant biosynthesis of canthaxanthin, indicating that the conversion of β-carotene to canthaxanthin, catalyzed by these CrtW β-carotene ketolase proteins, is more efficient, when compared to CrtO ketolase catalyzed reactions. Furthermore, enhanced activity of the CrtW ketolase proteins is also indicated by the observed increases in total carotenoid biosynthesis within these cultures. We believe this to be the case because comparison of total carotenoid production between *E. coli* strains harboring pAC-BETA alone, and in conjunction with CrtO type ketolase genes, to strains co-expressing pAC-BETA and CrtW ketolase genes, reveals increases of 2.5- to 13-fold. This again demonstrates the level of catalytic variation within the protein class.

### Functional Expression of Cyanobacterial β-Carotene Ketolase Genes in Zeaxanthin Accumulating *E. coli*

Zeaxanthin biosynthesis, within *E. coli*, was achieved via transformation with plasmid pAC-ZEAX (Sun et al., 1996). This enabled the biosynthesis of 65 $\mu g\,g^{-1}$ (197 $\mu g\,L^{-1}$) zeaxanthin. Heterologous expression of cyanobacterial CrtO β-carotene ketolase genes, within zeaxanthin accumulating *E. coli*, revealed that zeaxanthin is not a substrate for this class of protein, since zeaxanthin was the sole carotenoid compound produced (data not shown). Further, this also demonstrated that no investigated CrtO β-carotene ketolase protein is catalytically active enough for it to successfully compete with the constitutively expressed β-carotene hydroxylase, of pAC-ZEAX (derived from *Pantoea agglomerans* (formerly *Erwinia herbicola*), for the substrate, β-carotene.

It is widely accepted that the arabinose inducible pBAD promoter enables enhanced control of heterologous protein production within *E. coli* (Guzman et al., 1995), when compared to pTrc (IPTG inducible) expression systems (Kim and Keasling, 2001). To examine whether improved expression of the *Synechocystis* sp. PCC 6803 derived CrtO β-carotene ketolase protein would enhance carotenoid biosynthesis, this gene was expressed from the pBAD promoter of plasmid pBAD24. The result of this, within β-carotene accumulating *E. coli*, was the synthesis of 216 $\mu g\,g^{-1}$ (334 $\mu g\,L^{-1}$) echinenone and 146 $\mu g\,g^{-1}$ (226 $\mu g\,L^{-1}$) canthaxanthin, when expression was induced with 0.02% L-arabinose (final concentration), representing a 7.2-fold ($\mu g\,g^{-1}$ basis) increase in keto-carotenoid production. Within this, canthaxanthin increased from 6% to 40% of total keto carotenoids produced. Thus, despite the common reference to CrtO ketolase proteins as monoketolases, it is clear that they are in fact capable of enhanced production of di-keto-carotenoids, when heterologous expression is achieved in a suitable manner. Subsequently, it was hypothesized that if the hydroxylase gene is bi-functional, with respect to astaxanthin biosynthesis, then improved production of di-keto carotenoids should enable the CrtO ketolase to participate in a metabolic pathway culminating with the biosynthesis of astaxanthin. Following a brief investigation it was found that, within this system, carotenoid biosynthesis was maximal in the absence of induction, and while zeaxanthin remained the dominant carotenoid produced, canthaxanthin and astaxanthin were also synthesized at detectable levels (refer to supplementary material 4).

Expression of cyanobacterial CrtW ketolase genes, within zeaxanthin accumulating *E. coli*, revealed the universal capacity of cyanobacterial CrtW ketolases to participate in astaxanthin biosynthesis. With respect to this, the most productive CrtW ketolase was no longer that of *N. punctiforme* PCC 73102 (ZP_00345866.1), as was the case for canthaxanthin production (Fig. 2B), rather, the ketolase genes isolated from *Gloeobacter violaceus* PCC 7421 (NP_924674.1) and *Nostoc* sp. PCC 7120 (NP_487229.1), demonstrated enhanced productivity, catalyzing the synthesis of 1,234.5 $\mu g\,g^{-1}$ (1,407.2 $\mu g\,L^{-1}$) and 1,082.8 $\mu g\,g^{-1}$ (1,559.8 $\mu g\,L^{-1}$) astaxanthin, respectively (Fig. 3). Additionally, the *A. variabilis* ATCC 29413 (YP_32488.1) derived CrtW ketolase maintained enhanced activity, producing 922.7 $\mu g\,g^{-1}$ (1,190.5 $\mu g\,L^{-1}$) astaxanthin (Fig. 3). Significantly, within these systems, zeaxanthin and canthaxanthin were the only other carotenoid compounds detected, comprising 8% of total carotenoids, for each of these three systems, meaning that astaxanthin represented 92% of the total carotenoids produced.

The control afforded by the pTrc expression system was not sufficient, as although co-expression of CrtW ketolase genes, within zeaxanthin producing *E. coli*, predominantly resulted in the biosynthesis of astaxanthin, this was not

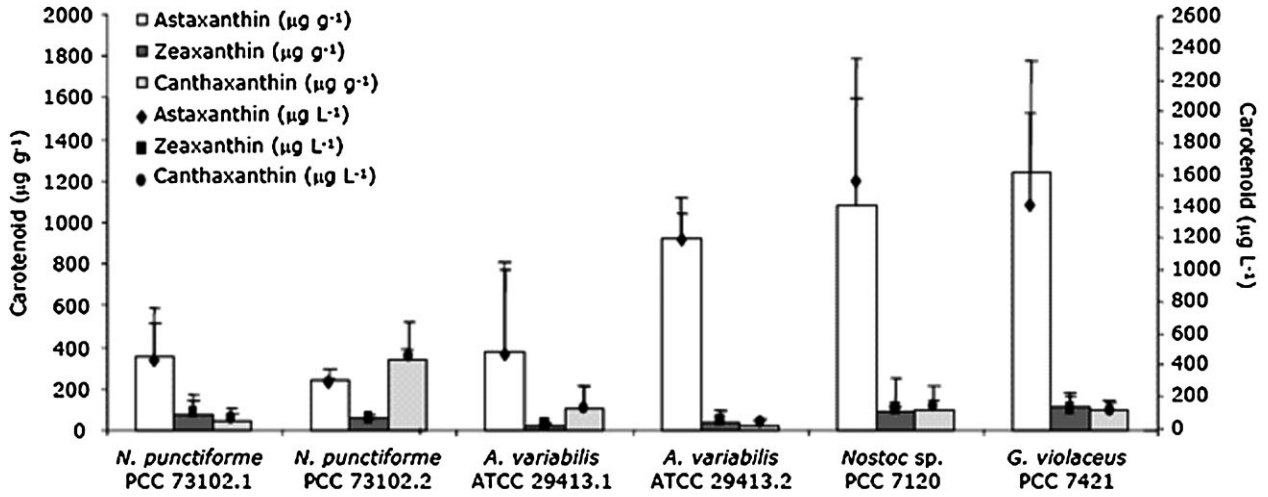


**Figure 3.** Carotenoid production due to heterologous expression of cyanobacterial CrtW β-carotene ketolase genes, within zeaxanthin accumulating *E. coli*. Values represent the mean ($n=6$) while error bars represent the standard deviation.

universal. This is because co-expression also resulted in the synthesis of the di-keto carotenoid, canthaxanthin as the main product. Such synthesis was also characterized by reduced synthesis of the hydroxylated carotenoid, zeaxanthin, and an absence of astaxanthin (data not shown). Due to this heterogeneity, the stringent pBAD promoter was employed for individual expression of three CrtW β-carotene ketolases.

The stringency of the pBAD expression system was evidenced here by a significant reduction in keto carotenoid biosynthesis, and a failure to produce astaxanthin, in the absence of induction (data not shown), while induction of heterologous (CrtW) gene expression, in zeaxanthin producing *E. coli*, allowed stable and reproducible biosynthesis of astaxanthin, for each of the investigated CrtW ketolases (Fig. 4). Specifically, the CrtW ketolases of *A. variabilis* ATCC 29413 (YP_324388.1) and *Nostoc* sp. PCC 7120 (NP_487229.1) produced 572 $\mu g\,g^{-1}$ (1,013 $\mu g\,L^{-1}$) and 960 $\mu g\,g^{-1}$ (1,421 $\mu g\,L^{-1}$) astaxanthin, while that from *N. punctiforme* PCC 73102 (ZP_00345866.1) remained reduced at 246 $\mu g\,g^{-1}$ (188 $\mu g\,L^{-1}$). Thus, although stable, it is apparent that the employed culture

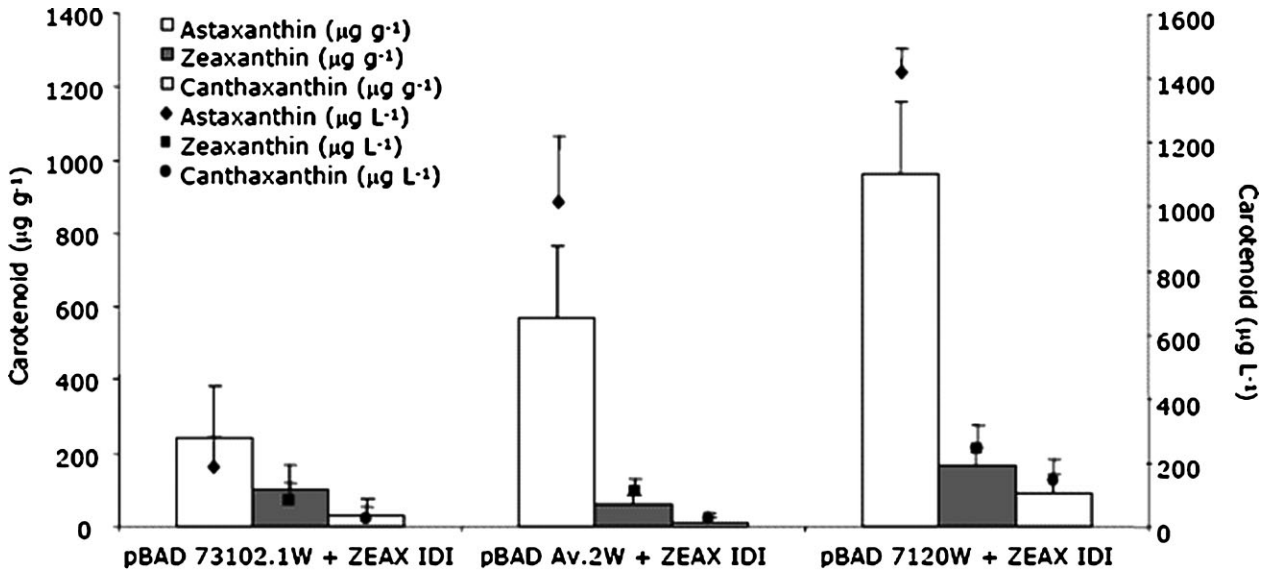


**Figure 4.** Astaxanthin production due to heterologous expression of cyanobacterial CrtW type β-carotene ketolase genes, from pBAD promoter, within zeaxanthin producing *E. coli*. Values represent the mean ($n=6$) while error bars represent the standard deviation.

conditions are not optimal, as astaxanthin biosynthesis was reduced both in quantity, and percentage of total carotenoids, when compared to the pTrc system (Fig. 3).

### Functional Expression of Cyanobacterial β-Carotene Hydroxylase Genes in β-Carotene Accumulating *E. coli*

In silico analysis of five cyanobacterial genomes revealed that putative β-carotene hydroxylases are less well represented within cyanobacteria, than are β-carotene ketolase genes, with just four putative β-hydroxylases being identified. Of these, one has been previously identified, derived from *Synechocystis* sp. PCC 6803 (Masamoto et al., 1998). Thus, this investigation identified three additional putative CrtR β-carotene hydroxylases. Functional characterization via heterologous expression confirmed the function of the aforementioned CrtR hydroxylase of *Synechocystis* sp. PCC 6803, and also confirmed the function of the novel CrtR hydroxylase isolated from *A. variabilis* ATCC 29413. Further, this investigation demonstrated that the putative β-carotene hydroxylase genes isolated from *N. punctiforme* PCC 73102 and *Nostoc* sp. PCC 7120 are not functional. Direct comparison of the two identified CrtR hydroxylase genes revealed that the *Synechocystis* sp. PCC 6803 gene catalyses the synthesis of 3.8-fold more zeaxanthin, than does that isolated from *A. variabilis* ATCC 29413, producing 69 $\mu g\,g^{-1}$, compared to 18 $\mu g\,g^{-1}$. For comparison, the previously characterized β-carotene hydroxylase of *E. herbicola* was isolated from plasmid pAC-ZEAX, and expressed within the same system. This revealed that the bacterial CrtZ β-carotene hydroxylase encoded a protein with a 4.0- and 15.6-fold enhanced catalytic activity, when compared to the two cyanobacterial constructs, producing 279 $\mu g\,g^{-1}$ zeaxanthin. It was also noted that expression of the *E. herbicola* CrtZ hydroxylase from the heterologous pTrc promoter increased total carotenoid biosynthesis by 4.3-fold, when compared to the productivity of pAC-ZEAX. This demonstrates that zeaxanthin biosynthesis can be enhanced via improved expression of the CrtZ β-carotene hydroxylase gene (refer to supplementary material 5).

### Creation of Dual Expression Vectors, for Enhanced Astaxanthin Production in *E. coli*

Following the creation of a stable pBAD based expression system, for astaxanthin biosynthesis, and the recognition that enhanced expression of a β-carotene hydroxylase increases total carotenoid production, it was hypothesized that the combination of these two factors may positively influence cellular production of astaxanthin. Dual expression vectors, which encode two promoters, have been described previously (Humphreys et al., 2002; McNally et al., 1988). Within this it was demonstrated that the application of multiple promoters can negate issues associated the more common bicistronic vectors, which encode several genes, expressed via a single promoter (Kim et al., 2004). In the creation of the current dual expression vectors, two basic configurations were investigated, namely, pTrc*CrtW*-pBAD*CrtZ*, and the alternate pTrc*CrtZ*-pBAD*CrtW*. To allow this, the CrtZ gene isolated from pAC-ZEAX (so derived from *P. agglomerans* (formerly *E. herbicola*)), was inserted, via PCR mediated cloning into pBAD24. Heterologous expression of this in β-carotene accumulating *E. coli*, resulted in the production of 160 $\mu g\,g^{-1}$ (382 $\mu g\,L^{-1}$) zeaxanthin.

The pTrc*CrtW*-pBAD*CrtZ* dual expression systems, in which the CrtW genes are isolated from *A. variabilis* ATCC 29413 (YP_324388.1), *Nostoc* sp. PCC 7120 (NP_487229.1), and *N. punctiforme* PCC 73102 (ZP_00345866.1), were expressed separately within β-carotene accumulating *E. coli*. This, in the absence of a chemical inducer, resulted in the stable biosynthesis of astaxanthin, with final productivity values of 687 $\mu g\,g^{-1}$ (1,492 $\mu g\,L^{-1}$), 1,993 $\mu g\,g^{-1}$ (2,885 $\mu g\,L^{-1}$), and 1,931 $\mu g\,g^{-1}$ (3,385 $\mu g\,L^{-1}$), respectively. Within these systems, zeaxanthin was the only other detected carotenoid, thus the produced astaxanthin represented 83.8%, 91.2%, and 87.4% of the total carotenoids produced. Figure 5 compares the single and dual expression systems. Within this system, astaxanthin biosynthesis is increased 1.2 (1.5), 2.1 (2.0), and 7.9 (18.0) fold on a $\mu g\,g^{-1}$ ($\mu g\,L^{-1}$) basis. Expression of carotenoid biosynthesis genes, within the alternate configuration (pTrcCrtZ-pBADCrtW) resulted in reduced astaxanthin yields, with increased concentrations of intermediate compounds. This suggests that appropriate expression levels for the β-carotene ketolase and hydroxylase genes are essential for efficient astaxanthin biosynthesis.

## Discussion

It is well known that in the biosynthesis of astaxanthin, proceeding from β-carotene, functional β-carotene ketolase, and β-hydroxylase proteins are required. Further, it is recognized that one of these proteins must be bi-functional, with respect to its substrate. Based on this, cyanobacterial carotenoid biosynthesis genes involved in these essential steps were investigated here. The result of this was the identification and functional characterization of 16 cyanobacterial genes. The heterologous expression of 12 β-carotene ketolase genes has furthered our understanding significantly, as in vivo comparison of several β-carotene ketolase proteins has been possible. This revealed that of the investigated CrtO β-carotene ketolase proteins, that derived from *Synechocystis* sp. PCC 6803 was the most active. Given that of the cyanobacterial strains investigated, *Synechocystis* sp. PCC 6803 is unique in that it contains only a single β-carotene ketolase protein within its genome, this is not surprising from an evolutionary standpoint. Improved expression of this CrtO ketolase, via application of the pBAD promoter, demonstrated that despite the common reference to CrtO ketolases as mono ketolases, efficient expression can enable enhanced di-keto activity.

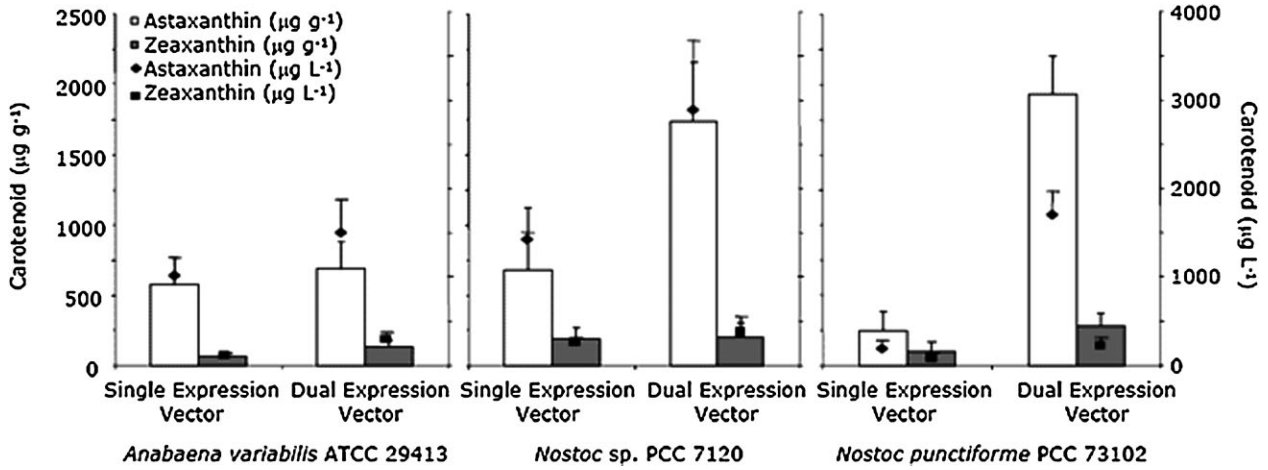


**Figure 5.** Comparison of astaxanthin biosynthesis via pBAD based single, and Trc*CrtW*-pBAD*CrtZ* dual expression vectors, within *E. coli* strain TOP10. Values represent the mean ($n = 6$) while error bars represent the standard deviation.

This suggests that rather than the mono-ketolase nature of this protein class being due to a functional restriction, it is in fact due to the reduced catalytic activity of these proteins. This is because, if the catalytic activity of the encoded CrtO ketolase is below that of the lycopene cyclase, the rate of β-carotene synthesis will be greater than the rate of β-carotene metabolism. Therefore, the substrate pool for the β-carotene ketolase will be continually diluted. The result of this will be the predominant conversion of β-carotene to echinenone, rather than the conversion of echinenone to canthaxanthin. Reported directed evolution studies further support this (Tang et al., 2007).

Studies focusing on CrtW β-carotene ketolase proteins resulted in the functional characterization of six CrtW β-carotene ketolases, three of which were novel. In vivo comparison demonstrated the significant catalytic variation within this class of protein, evidenced here by variation of up to 5.3 (8.0) fold on a $\mu g\, g^{-1}$ ($\mu g\, L^{-1}$) basis. Additionally, this investigation also demonstrated that total carotenoid biosynthesis could be enhanced by as much as 13-fold, simply by the heterologous expression of a CrtW type β-carotene ketolase gene. Co-expression of the CrtZ β-carotene hydroxylase derived from pAC-ZEAX also increased total biosynthesis significantly. This suggests the presence of a regulatory mechanism, possibly feedback inhibition, within the *Erwinia herbicola* carotenoid biosynthetic operon, as maximal increases were obtained by the conversion of β-carotene (native to the biosynthetic operon) to canthaxanthin, a foreign carotenoid. Regardless, it is apparent that deregulation of the carotenoid biosynthetic operon can enhance productivity.

Heterologous expression of target ketolase genes, within zeaxanthin accumulating *E. coli*, allowed investigations to focus on the function of β-carotene ketolase proteins within astaxanthin biosynthesis. Improved expression of the *Synechocystis* sp. PCC 6803 CrtO ketolase gene demonstrated the capacity of this protein type to function in astaxanthin biosynthesis, albeit at trace levels. Additionally, this study demonstrated that all investigated CrtW ketolases are able to function efficiently in astaxanthin biosynthesis. More importantly, this investigation also enabled the direct in vivo comparison of catalytic activity for astaxanthin biosynthesis, enabling robust candidate gene selection. Within this study, differences in the relative catalytic activity of investigated CrtW ketolases were evident with respect to canthaxanthin and astaxanthin biosynthesis. The most noticeable example was the CrtW ketolase isolated from *N. punctiforme* PCC 73102 (ZP_00345866.1). This suggests that in canthaxanthin and astaxanthin biosynthesis, two different substrates are utilized. It is important to consider that in canthaxanthin, zeaxanthin, and astaxanthin biosynthesis the carotenoid molecule should not be considered a single compound, rather it should be considered the product of its two β-ionone rings, as a protein will not likely recognize an entire carotenoid molecule. This is evidenced by the catalytic activity of β-hydroxylase proteins that function in the synthesis of both lutein and zeaxanthin (Kim and DellaPenna, 2006; Tian and DellaPenna, 2004). In canthaxanthin biosynthesis, β-carotene is the only substrate available, while in astaxanthin biosynthesis both β-carotene and zeaxanthin are theoretically available for metabolism by the ketolase. In our experimental system, the suggestion is that astaxanthin biosynthesis proceeds from β-carotene through hydroxylation first, and then onto ketolation. This is evidenced by the change in catalytic efficiency when the *N. punctiforme* PCC 73102 CrtW is expressed in β-carotene and zeaxanthin accumulating cells, and suggests that within the catalytic site of the CrtW ketolase, a mutation has taken place that reduces its zeaxanthin utilization capacity. Such mutations

have been identified previously (Tao et al., 2006; Ye et al., 2006).

Astaxanthin biosynthesis was further improved via the creation of several novel dual expression vectors. This demonstrated that enhanced expression of the *E. herbicola* derived CrtZ hydroxylase could increase astaxanthin production, while also revealing that the correct expression profile for these two proteins is essential to astaxanthin yield and purity. Furthermore, this also demonstrated the restricted nature of carotenoid biosynthesis due to pAC-BETA expression alone, as additional co-expression of pTrc-*CrtW*-pBAD-*CrtZ* increased carotenoid biosynthesis by up to 23.5 (17.6) fold on a $\mu g\,g^{-1}$ ($\mu g\,L^{-1}$) basis.

Due to the successful biosynthesis of astaxanthin, within the presented systems, one of two hypotheses may be proposed. Either the investigated cyanobacterial CrtW ketolases are able to successfully compete with the β-hydroxylase, in all instances, with respect to the substrate β-carotene, converting this to canthaxanthin. Implicit to this is the bi-functional nature of the *E. herbicola* β-hydroxylase. Or alternatively, the cyanobacterial CrtW type ketolases are not able to compete with the β-hydroxylase, but are bi-functional with regards to their substrate. Based on this analysis alone it is not possible to discount or confirm either possibility. This is because a comparison of carotenoid biosynthesis yields suggests that catalytic efficiency does not influence the capacity of a ketolase to function in astaxanthin biosynthesis, determined by the relative productivities of most active CrtO, and least active CrtW ketolases.

Therefore, this suggests that the apparent disparity between CrtO and CrtW type ketolases, with respect to astaxanthin biosynthesis, is not related to catalytic activity, rather it is a function of differences in substrate specificity. This suggests that within this system, astaxanthin biosynthesis proceeds via hydroxylation and then ketolation. Further evidence in support of this route is provided by a comparison of carotenoid biosynthesis productivities for the CrtW ketolases, when expressed in β-carotene and zeaxanthin accumulating *E. coli*. This is because, when the pTrc based expression system was employed for astaxanthin biosynthesis, an absence of astaxanthin biosynthesis was additionally characterized by the synthesis of canthaxanthin. If the β-hydroxylase was bi-functional, this would not be possible. Thus it is suggested that the ketolase gene is bi-functional, rather than instability in the pAC-ZEAX plasmid, as in no other investigations was any instability apparent.

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