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Cloning of two carotenoid ketolase genes from *Nostoc punctiforme* for the heterologous production of canthaxanthin and astaxanthin

Sabine Steiger & Gerhard Sandmann*
Biosynthesis Group, Botanical Institute, Goethe University, P.O. Box 111932, D-60054 Frankfurt, Germany
**Author for correspondence (Fax: +49 69 789 24730; E-mail: sandmann@em.uni-frankfurt.de)*

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Abstract

For the heterologous synthesis of keto-carotenoids such as astaxanthin, two carotenoid ketolase genes *crtW38* and *crtW148*, were cloned from the cyanobacterium, *Nostoc punctiforme* PCC 73102 and functionally characterized. Upon expression in *Escherichia coli*, both genes mediated the conversion of β-carotene to canthaxanthin. However in a zeaxanthin-producing *E. coli*, only the gene product of *crtW148* introduced 4-keto groups into the 3,3′-dihydroxy carotenoid zeaxanthin yielding astaxanthin. The gene product of *crtW38* was unable to catalyze this reaction. Both ketolases differ in their interaction with a hydroxylase in the biosynthetic pathway from β-carotene to astaxanthin.

Introduction

The economic interest on carotenoids is based on their use as food and feed colorants. In particular, keto-carotenoids like canthaxanthin (β, β-carotene-4,4′-dione) and astaxanthin (3,3′-dihydroxy-β, β-carotene-4,4′-dione) are important feed supplements in the farming of trout and salmon (Meyers 1994). While a considerable number of species forming canthaxanthin or the mono keto derivative echinenone exist, only a few astaxanthin forming organisms including the green alga *Haematococcus pluvialis* and the fungus *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) are known (Goodwin 1980, Johnson & An 1991, Lorenz & Cysewski 2000).

The final steps in astaxanthin formation are hydroxylation and ketolation of β-carotene (Figure 1). In *X. dendrorhous*, a single enzyme carries out this reaction sequence. In all other known cases the modification reactions of β-carotene are independent. Genes for CrtW type diketolases were cloned and functionally characterized from the bacteria *Agrobacterium aurantiacum* (Misawa *et al.* 1995) and *Paracoccus marcusii* (Harker & Hirschberg 1999) and also from *H. pluvialis* (Kajiwara *et al.* 1995), whereas in the cyanobacterium *Synechocystis* PCC 6803 a CrtO type monoketolase was identified (Fernández-Gonzalez *et al.* 1997).

For the synthesis of astaxanthin in species other than *X. dendrorhous*, a β-carotene 4-ketolase and a 3-hydroxylase have to interact efficiently. Enzymatic studies with the ketolase indicate that zeaxanthin is poorly converted by the ketolase from *A. aurantiacum* (Fraser *et al.* 1998). Another important feature to take into account in astaxanthin biosynthesis is that only specific hydroxylase enzymes, for example CrtZ from *Erwinia uredovora* (Breitenbach *et al.* 1996) and *A. aurantiacum* (Misawa *et al.* 1995), are able to modify a β-carotene molecule already carrying a keto group efficiently. The structurally different hydroxylases from cyanobacteria are not able to accept echinenone or canthaxanthin as substrates (Albrecht *et al.* 2001). These examples demonstrate that the co-operation of appropriate hydroxylase and ketolase enzymes are crucial for the synthesis of substantial amounts of astaxanthin. Therefore, it is important to find and identify ketolases which are able to interact with many endogenous hydroxylases of potential heterologous hosts for astaxanthin biosynthesis. Suitable sources for these ketolases are cyanobacteria which

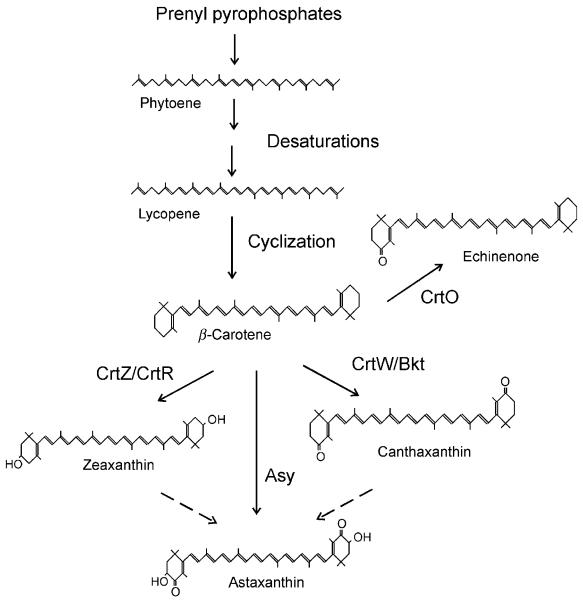

Fig. 1. Carotenoid biosynthetic pathway to 3-hydroxy and 4-keto derivatives of β-carotene. The different gene products able to catalyze the steps from β-carotene are specified. Dotted arrows indicate a hydroxylation of canthaxanthin or ketolation of zeaxanthin by enzymes able to co-operate with each other.

are able to synthesize canthaxanthin. From some of them including *Nostoc punctiforme* PCC 73102, sequence information of the genome is partial or complete available at www.jgi.doe.gov. The species mentioned was chosen to search for putative ketolase genes and to demonstrate the metabolic function of the corresponding enzymes in the formation of canthaxanthin and astaxanthin.

Materials and methods

Growth and genetic manipulations

Nostoc punctiforme PCC 73102 was grown in mineral BG11 medium including $NaNO_3$ (5.8 mM) as nitrogen source (Rippka *et al.* 1979). For the isolation of genomic DNA, cells were harvested, immediately treated with lysozyme (10 mg ml^{-1} for 1 h) and genomic DNA was isolated with the GenElute plant genomic DNA kit (Sigma-Aldrich, St. Louis, MO). ORF148 (765 bp) and ORF38 (786 bp) were amplified by PCR under standard conditions using oligonucleotides 148-start (5′-ATGATCCAGTTAGAACAACCAC-3′), 148-end (5′-CTATTTTGCTTTCGTAAATTCTGG-3′) or 38-start (5′-ATGAATTTTTGTGATAAACCAGTTAG-3′) and 38-end (5′-ACGAATTGGTTACTGAATTGTTG-3′). The PCR products were ligated into *Xcm*I site of pMON 38201 (Borovkov & Rivkin 1997). After restriction of the constructs with *Hin*dIII, the resulting fragments were cloned in the *Hin*dIII digested and dephosphorylated vector pPQE32 (pQE32 from Qiagen, Hilden, Germany, modified according to Verdoes *et al.* 1999).

Carotenoid production in E. coli *and analysis*

Functional characterization of the gene products from ORF148 and ORF38 was done by transforming plasmids pPQE32-148 and pPQE32-38 in the β-carotene producing *E. coli* JM101/pACCAR16 ΔcrtX or the zeaxanthin producing transformant JM101/pACCAR25ΔcrtX (Misawa *et al.* 1995). Transformants were cultivated in LB medium (Sambrook *et al.* 1989) containing chloramphenicol (0.034 mg ml^{-1}) and ampicillin (0.1 mg ml^{-1}) in the dark at 28 °C. After 16 to 48 h, carotenoids were extracted with methanol at 60 °C for 20 min and partitioned into 50% ether in petrol. The extracts were separated by HPLC on a HyPurity C18, 5 μm column (Hypersil, Kleinostheim, Germany) with acetonitril/methanol/2-propanol (85:10:5, by vol.) as mobile phase. The absorbance spectra of the carotenoids were recorded on-line with a Waters 440 photodiode array detector.

Results and discussion

In the sequence database of *N. punctiforme* PCC 73102 (www.jgi.doe.gov) two open reading frames, ORF148 and ORF38, with homologies to ketolases of the CrtW type were found. They show homology to the functionally well characterized ketolases from *Agrobacterium aurantiacum* and *Haematococcus pluvialis* (Figure 2) with identities on the amino acids level from 33% to 36%. The conserved domains present in both open reading frames are also found in the putative ketolase from the cyanobacterium *Nostoc* (also named *Anabaena* in some publications) PCC 7120. Conserved iron binding motifs are underlined in Figure 2.

Both reading frames were cloned into the expression vector pPQE32 as an in-frame fusion to the *lacZ* gene, resulting in plasmids pPQE32-38 and pPQE32-148, which are used for the characterization of the gene products by functional complementation

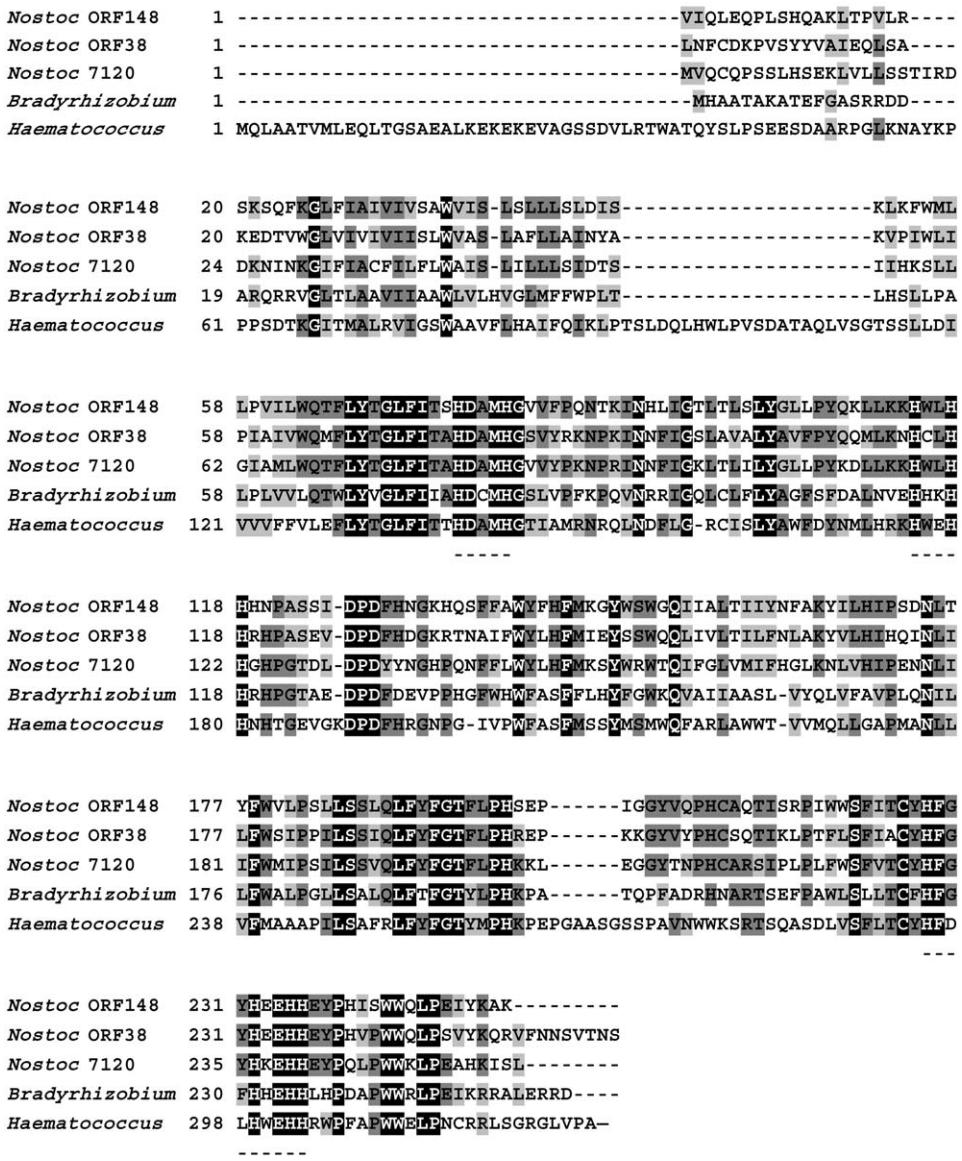

Fig. 2. Amino acid sequence comparison of ORF38 and ORF148 from *Nostoc punctiforme* PCC 73102 with ketolases of the crtW type: alr3189 from *Nostoc* PCC 7120, ORS 278 from *Bradyrhizobium* sp. and CrtW from *Haematococcus pluvialis.*

in *E. coli* as heterologous host. The HPLC profiles of the carotenoids from the complemented cells with a β-carotene background shown in Figures 3A and B indicate that both gene products are able to convert β-carotene (peak 3) via echinenone (peak 2) to canthaxantin (peak 1). In the transformant with pPQE32-148, the diketo-carotenoid canthaxanthin represents 81% of the total carotenoid and reached 40% in the transformant containing plasmid pPQE32-38. In both complementation reactions the amount of the monoketo derivative echinenone is about 4%.

To investigate whether the ketolases are also able to synthesize astaxanthin by the conversion of the 3,3′-dihydroxylated β-carotene derivative zeaxanthin, pPQE32-148 and pPQE32-38 were transformed in a zeaxanthin producing *E. coli* transformant. In the complementation reaction with pPQE32-38 (trace C) only the substrate zeaxanthin (peak 4) (85%) and trace amounts of echinenone were produced. However, in the complementation with pPQE32-148 (trace D), echinenone, canthaxanthin and astaxanthin (peak 5) are found in large amounts. Astaxanthin repres-

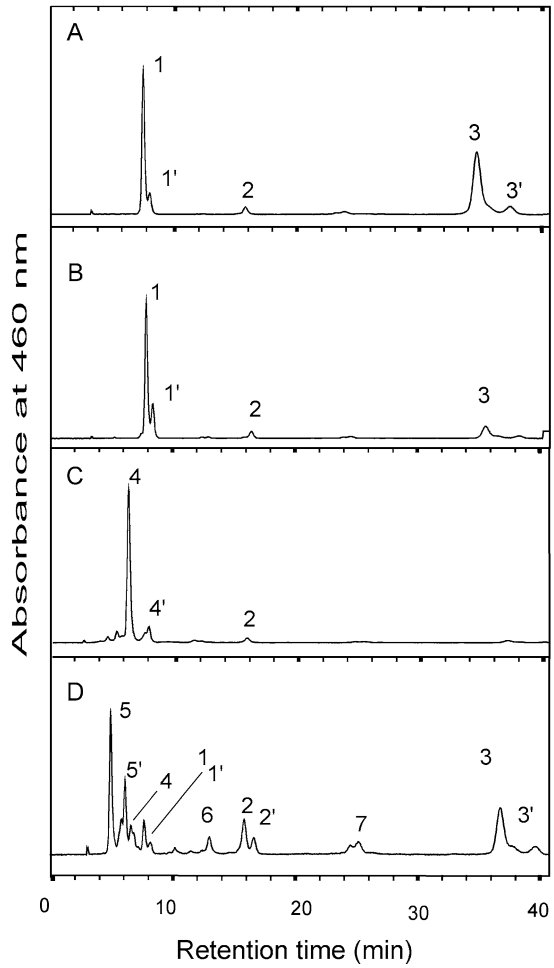

Fig. 3. HPLC separation of extracts from complementation reactions of a β-carotene producing *E. coli* transformant with pPQE32-38 (A), pPQE32-148 (B) or an *E. coli* with a zeaxanthin background co-transformed with pPQE32-38 (C) or pPQE32-148 (D). Peak 1and 1′ canthaxanthin isomers, 2 echinenone, 3 and 3′ β-carotene isomers, 4 and 4′ zeaxanthin isomers, 5 and 5′ astaxanthin isomers, 3′-HO-echinenone and 7 neurosporene. Carotenoids were identified by there retention times and spectra and co-chromatography using authentic standards.

ents with 50% the major carotenoid, the intermediates of the astaxanthin pathway echinenone and canthaxanthin are present with 12% and 8% of the total carotenoids. In addition, 3′-hydroxy-echinenone (peak 6) was detected together with the β-carotene precursor neurosporene (peak 7).

Both open reading frames found in *N. punctiforme* PCC 73102 encode for a functional β-carotene ketolase and are able to carry out the ketolation of β-carotene shown in the bottom part of Figure 4. While the gene product of ORF38 is restricted to

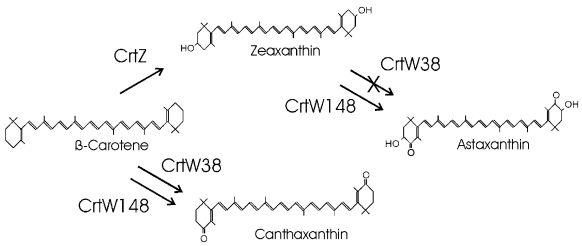

Fig. 4. Reactions mediated by the ketolases CrtW38 and CrtW148 from *Nostoc punctiforme* PCC 73102.

this conversion of β-carotene to echinenone and canthaxanthin, ORF148 encodes a ketolase which shows an excellent interaction with hydroxylase enzymes from the bacterial CrtZ type resulting in the synthesis of substantial amounts of astaxanthin (Figure 4 top part). Other characterized ketolases like those from *A. aurantiacum* (Fraser *et al.* 1998) and *H. pluvialis* (Breitenbach *et al.* 1996) are very poor in the conversion of zeaxanthin to astaxanthin. In these species, the preferred reaction sequence is ketolation first and then hydroxylation. CrtW148 from *N. punctiforme* is the only ketolase with an established efficient conversion potential for hydroxy β-carotene derivatives. Therefore, this gene is highly suitable for heterologous production of astaxanthin in a zeaxanthin-producing host.

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