COMMUNICATION TO THE EDITOR

Cloning and Characterization of the Astaxanthin Biosynthetic Gene Encoding Phytoene Desaturase of *Xanthophyllomyces dendrorhous*

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Abstract: The first carotenoid biosynthetic gene from the basidiomycetous yeast *Xanthophyllomyces dendrorhous* was isolated by heterologous complementation in *Escherichia coli.* The isolated gene, denominated as *crtI,* was found to encode for phytoene desaturase. The coding region is interrupted by 11 introns. The deduced amino acid sequence showed significant homology with its bacterial and eukaryotic counterparts, especially those of fungal origin. A plasmid containing the geranylgeranyl diphosphate synthase and phytoene synthase encoding genes from *Erwinia uredovora* was introduced in *E. coli* together with the phytoene desaturase encoding cDNA from *X. dendrorhous.* As a result, lycopene accumulation was observed in these transformants. We conclude that in *X. dendrorhous* the four desaturase steps, by which phytoene is converted into lycopene, are carried out by a single gene product. *Biotechnol Bioeng* **63**: 750–755, 1999.
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INTRODUCTION

More than 600 carotenoids have been identified in nature which display a large variety of biological functions depending on their structure, chemical, and physical properties. Details on the structure, function, and biosynthesis of carotenoids have recently been reviewed (Britton, 1995; Olson and Krinsky, 1995; Armstrong and Hearst, 1996; Demming-Adams et al., 1996; Johnson and Schroeder, 1995; Parker, 1996; Mayne, 1996). Carotenoid biosynthesis is found in bacteria, fungi, algae, and plants. A few of the identified carotenoids are used industrially for food coloring, animal feeding, pharmaceuticals, and cosmetics (Johnson and Schroeder, 1995).

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Carotenoids can be synthesized chemically or extracted from biological sources (Johnson and Schroeder, 1995). These latter ones can be used as natural colorants in food and feed industry. Although much interest and effort has been devoted to the use of biological sources for industrially important carotenoids, only biological production of β-carotene and astaxanthin has been reported (Johnson and Schroeder, 1995). Besides the green alga *Haematococcus pluvialis* and crustacean extracts, the red basidiomycetous yeast *Xanthophyllomyces dendrorhous* (the perfect state of *Phaffia rhodozyma;* Golubev, 1995) has been identified as the best biological source of astaxanthin (Johnson and Schroeder, 1995, 1996).

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is widely distributed in nature; also in organisms which are unable to synthesize carotenoids in vivo. Astaxanthin produced primarily by phytoplankton contributes to the distinct red or pinkish color of some marine animals (i.e. salmons, crustaceans, and birds). Presently, chemically synthesized astaxanthin is added to the feed for the pigmentation of farm-raised salmon and crustaceans. The potential market in aquaculture, the health-promoting role of carotenoids (Johnson and Schroeder, 1995, 1996), and the preference of consumers for natural products have resulted in considerable commercial interest for developing *X. dendrorhous* as competitive biological sources of astaxanthin.

A scheme, based on mutants affecting carotenoid biosynthesis and the use of specific inhibitors of biosynthetic enzymes, for the astaxanthin biosynthetic pathway in *X. dendrorhous* was proposed by Andrewes et al. (1976). Metabolic engineering of this multi-enzyme biosynthetic pathway using recombinant DNA technology is one of the approaches to improve the production level of astaxanthin, which is rather low (400 μg/g dry weight) in wild type

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strains. For such an approach a transformation system and the availability of the genes encoding astaxanthin biosynthetic enzymes are essential. Recently, an efficient transformation system for *X. dendrorhous* has been developed (Wery et al., 1997, 1998). However, the molecular basis of the astaxanthin production in *X. dendrorhous* is still unresolved.

In this paper, we describe the isolation of the first gene from *X. dendrorhous* involved in astaxanthin biosynthesis by a heterologous complementation approach in *Escherichia coli.* The function of the cDNA was determined through chromatographic analysis of pigments in different carotenogenic *E. coli* transformants.

MATERIALS AND METHODS

General Procedures

Plasmid preparations, transformations, and other standard molecular biology techniques were carried out as described (Sambrook et al., 1989). Determination of a nucleotide sequence was performed using a Taq DYE Primer Cycle Sequencing kit (Applied Biosystems). The plasmid pBluescript was used for subcloning experiments.

Strains and Cultivation Conditions

All strains and plasmids used in this study are outlined in Table I. The construction of a genomic library of *X. dendrorhous* CBS 6938 in a cosmid vector was described previously (Verdoes et al., 1997). The cDNA library of an overproducing mutant of *X. dendrorhous* CBS6938 was constructed in the Uni-ZAP XR vector (Verdoes et al., 1997). The carotenogenic *E. coli* to be used in the complemention experiments was made by the introduction of the plasmid pACCAR25ΔcrtI in strain XL-Blue-MRF′. This plasmid contains all *Erwinia uredovora* genes involved in the biosynthesis of the yellow/orange carotenoid zeaxanthin diglucoside (Misawa et al., 1990) with the exception of the phytoene desaturase encoding gene (*crtI*). The cDNA library of *X. dendrorhous* was introduced in *E. coli*[pACCAR25ΔcrtI] and plated on LB-agar plates containing ampicillin, chloramphenicol and IPTG. The plates were incubated overnight at 37°C, and incubation was continued at room temperature until proper pigmentation of the transformants occurred. A phytoene accumulating *E. coli* strain was made by the introduction of the plasmid pACCRT-EB in strain JM101, further indicated as JM101[pACCRT-EB]. This plasmid contains the genes encoding GGPP synthase and phytoene synthase from *E. uredovora* (Misawa et al., 1995).

Analysis of Carotenoid Production

For complementation experiments the *E. coli* strain JM101 was grown for 48 h at 28°C. The plasmid pPR*pd*1 used for cotransformation together with either pACCRT-EB (Linden et al., 1991), for synthesis of phytoene, or pACCAR25ΔcrtI with a deletion of *crtI* in the *Erwinia* carotenoid gene cluster. As control an empty cloning vector pUC18 was used. Carotenoids were extracted from freeze-dried cell either with hot methanol at 60°C for 20 min when the yellow zeaxanthin glucoside was expected or with hot acetone at 55°C in case of carotenoids. The carotenoids were partitioned into 50% ether in petroleum and separated, identified, and quantitated by HPLC. The HPLC system involved a 25-cm Nucleosil C18, 3μ column with acetonitrile/methanol/2-propanol (85:10:5, v/v/v) as eluent at a flow rate of 1 mL/min. Absorbance spectra were recorded on-line from the elution peaks with a Kontron Model 440 photodiode array detector. Identification was by spectral characteristics and by cochromatography with authentic standards.

Table I. Strains and plasmids used in this study.

Strain	Genotype	References
E. coli		
XL-Blue-MRF′	*Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1*	
JM101	*recA1 gyrA96 relA1 lac*[F′ *proAB laq*^{q}*ZΔM15* T*n10*	Gibco BRL
	supE thi Δ(lac-proAB) F′[*traD36 proAB*$^+$ *lac1*^{q}*lacZΔM15*]	Messing (1979)
X. dendrorhous (P. rhodozyma): CBS 6938		

Plasmids	Insert	
pUC19	Cloning vector	Yanisch-Perron et al. (1985)
Uni-ZAP XR	Cloning vector	Strategene^R
pACYC184	Cloning vector	Rose (1988)
pMT6	Cloning vector	Breter et al. (1987)
pACCAR25ΔcrtI	Fragment carrying genes encoding geranylgeranyl diphosphate (GGPP) synthase *(crtE)*, phytoene synthase *(crtB)*, lycopene cyclase *(crtY)*, β-carotene hydroxylase *(crtX)*, zeaxanthin glycosylase *(crtZ)* of *E. uredovora* in pACYC184	Linden et al. (1991)
pACCRT-EB	Fragment carrying *crtE* and *crtB* genes *E. uredovora* in pACYC184	Linden et al. (1991)
pPRcos	Clone of genomic library of *X. dendrorhous* in pMT6	Verdoes et al. (1997)
pPRpd	Fragment carrying cDNA encoding phytoene synthase of *X. dendrorhous*	This study

RESULTS AND DISCUSSION

Cloning of a cDNA Coding for Phytoene Desaturase

To clone the phytoene desaturase encoding gene from *X. dendrorhous*, a cDNA library was introduced in a genetically engineered, phytoene accumulating *E. coli* strain. This strain was constructed by the introduction of the plasmid pACCAR25ΔcrtI (Linden et al., 1991) in strain XL-Blue-MRF′. This plasmid contains all *E. uredovora* genes involved in the biosynthesis of the yellow/orange carotenoid zeaxanthin diglucoside (Misawa et al., 1990) with the exception of the phytoene desaturase encoding gene (*crtI*). The selection method is based on the assumption that the color of XL[pACCAR25ΔcrtI] containing a cDNA encoding phytoene desaturase will change from white (phytoene) into yellow/orange (zeaxanthin diglucoside) as the *E. uredovora* biosynthetic pathway is restored. Two of about 14,000 colonies were found to have a yellow/orange color. The corresponding plasmids called pPRpd1 and pPRpd2 were separated from the complementation plasmid pACCAR25ΔcrtI and used for further analysis.

All transformants obtained after reintroduction of both plasmids in XL[pACCAR25ΔcrtI] had a yellow/orange phenotype. The carotenoids from these transformants were extracted and the composition was analyzed by HPLC. It was found that complementation resulted in the formation of the cyclic carotenoids zeaxanthin and zeaxanthin diglucoside (67 μg/g dry weight, Table II). In control complementation experiments using pUC18 no conversion of the substrate phytoene was detected (Table II).

Nucleotide Sequence of the Phytoene Desaturase Encoding Gene of *X. dendrorhous*

Restriction analysis of pPRpd1 and pPRpd2 indicated that both carried an insert of 2 kb and that the inserts were almost identical (not shown). Both cDNA were sequenced and small differences in length at both the 5′- and 3′-ends of the untranslated region were observed (Fig. 1). The deduced amino acid sequence was compared with EMBL protein data base and was found to have moderate but significant homology with other phytoene desaturase sequences. The *X. dendrorhous* phytoene desaturase was found to have 582 amino acids residues with a calculated mass of 65.1 kDa.

To determine the nucleotide sequence of the phytoene desaturase encoding gene and its flanking regions the cDNA fragment was used to screen a cosmid library of *X. dendrorhous.* One positive cosmid called pPR*pd*cos1 was detected by analyzing 6000 clones. Southern blot analysis of several restriction enzyme digests of this cosmid indicated that a 3-kb *Hin*dIII and a 4-kb *Hin*dIII–*BamH*I fragment hybridized strongly with the probe. These fragments, which were also present on blots containing *X. dendrorhous* genomic DNA digested with the same enzymes, were subcloned into pUC19 and used for sequence analysis. To prove that both fragments were contiguous, the nucleotide sequence of the cosmid clone was determined using oligonucleotides near the bounderies of the *Hin*dIII restriction site.

Comparison of the nucleotide sequence of both the genomic and the cDNA sequences revealed that the coding region of *X. dendrorhous* gene encoding phytoene desaturase, compared to phytoene desaturase encoding genes from other organisms, is interrupted by an extremely high number of intervening sequences (Fig. 1). The intron sizes range between 74 and 176 nucleotides and are positioned at the N-terminal part of the gene. These characteristics and the intron structure are consistent with data concerning the genomic structure in other *X. dendrorhous* genes (Wery et al., 1996; Verdoes et al., 1997). Compared to genes of *X. dendrorhous* encoding actin (Wery et al., 1996) and glyceraldehyde-3-phosphate dehydrogenase (Verdoes et al., 1997), the codon usage in the gene encoding phytoene desaturase is less biased. With the exception of ATA, all triplets are used in the phytoene desaturase encoding gene of *X. dendrorhous.* In contrast to the strong bias observed in bacterial carotenoid genes (Matsumura et al., 1997), also in *X. dendrorhous* the triplets CTA, GTA, and TTA are used albeit with a low frequency. The observed differences in codon usage in the cloned *X. dendrorhous* genes might be the result of the differences in expression levels or in genetic evolutionary origin. The amino acid sequence of phytoene desaturase from *X. dendrorhous* was aligned with all available phytoene desaturase protein sequences. The highest homology between phytoene desaturase proteins was found in the region forming dinucleotides (NAD(H)/NADP(H) or

Table II. Formation of carotenoids in *E. coli* transformants.

Plasmids	Carotenoids[a] (γg/g dry weight)			
	Phytoene	Lycopene	Zeaxanthin	Zeaxanthin–glucoside
pACCAR25ΔcrtI + pUC18	285.4	nd	nd	nd
pACCAR25ΔcrtI + pPRpd1	114.5	0	50.3	17.4
pACCRT-EB + pUC18	365.8	nd		
pACCRT-EB + pPRpd1	183.5	144.3		

[a]Average from 3 determinations; nd, not detected.

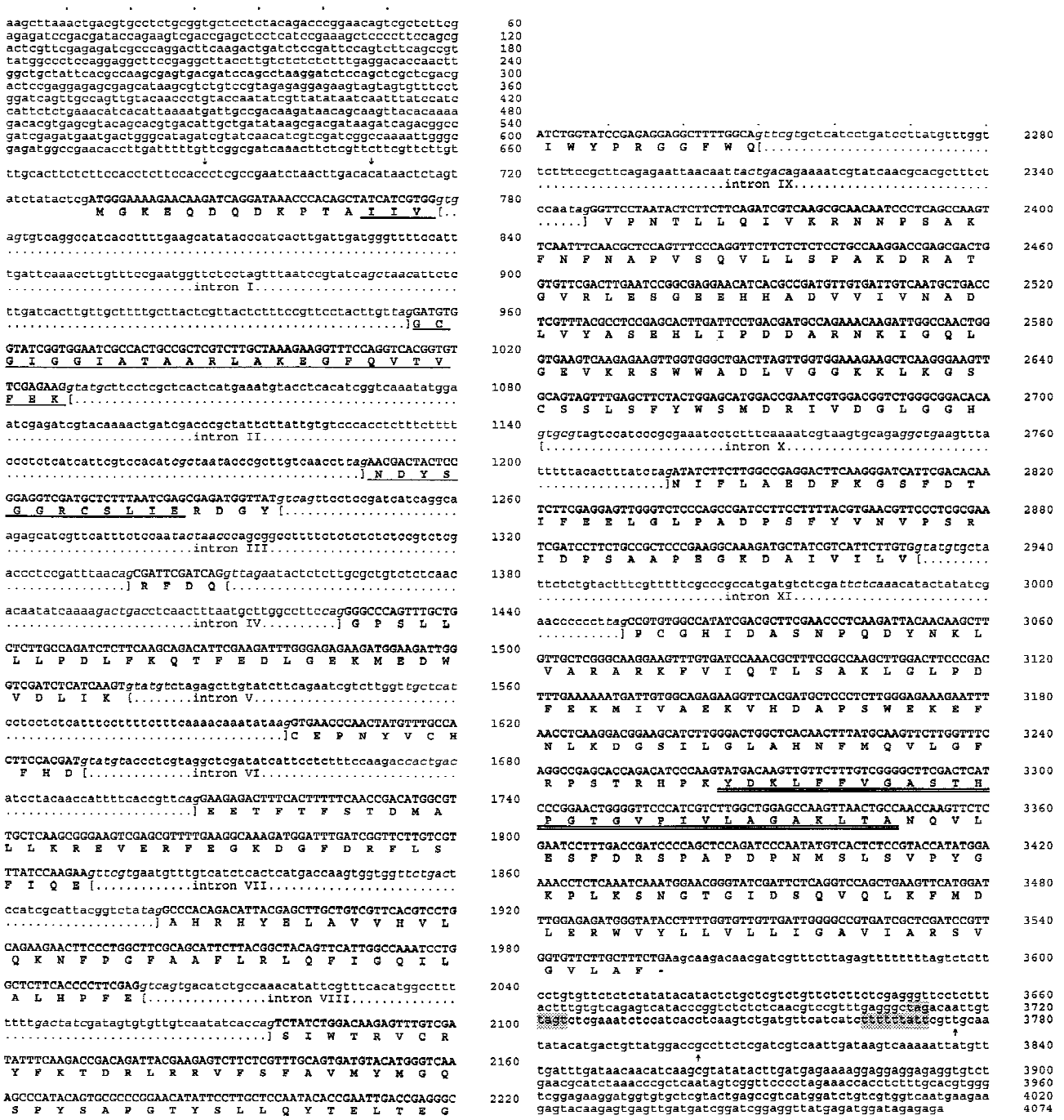

Figure 1. The nucleotide sequence of the *X. dendrorhous* phytoene desaturase encoding gene (*crtI*) and its flanking regions. The sequence is numbered from the first nucleotide. The deduced amino acid sequence is shown below the nucleotide sequence. The regions homologous to the dinucleotides (NAD(H)/NADP(H) or FAD(H))-binding domain (Pecker et al., 1992: Linden et al., 1994) and the carotenoid-binding domain (Armstrong et al., 1989) are indicated respectively by underlining and double underlining. The 5′ and 3′ intron splice junctions and putative 3′ internal splice signal sequence are indicated by italics. The putative polyadenylation signals are indicated by red lines. The first and last nucleotide of the two cDNA clones analyzed is indicated by ↓ and ↑, respectively. The nucleotide sequence has been submitted to the EMBL Data Bank with accession number Y15007.

FAD(H))-binding domain (Pecker et al., 1992; Linden et al., 1994) and a postulated sequence to be the carotenoid-binding domain (Armstrong et al., 1989) (Fig. 1). This latter region is also found in the phytoene desaturase sequences of other fungi and bacteria but not in cyanobacteria and plants. The deduced phylogenetic tree (Fig. 2) indicates that within phylogenetic clusters formed by plants, eubacteria, fungi, and cyanobacteria, a clear homology is found. Fungal phytoene desaturases are more related to the eubacterial phytoene desaturase proteins than to the phytoene desaturase proteins within the clusters formed by plants and cyanobacteria. The phytoene desaturase protein of the heterobasidio-

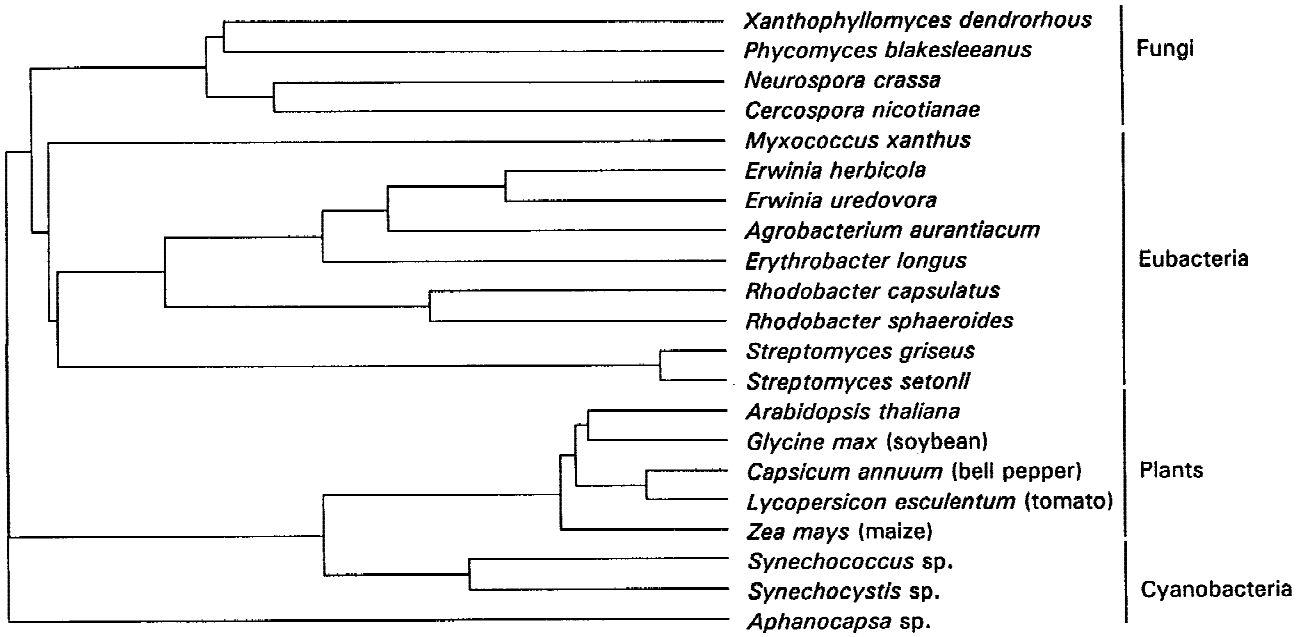

Figure 2. Dendogram of the alignment of the deduced sequences of 21 phytoene desaturases from different organisms. Amino acid sequences were analyzed using PC/GENE programs package.

mycetous yeast *X. dendrorhous* forms a cluster with phytoene desaturases from other fungi. The distantly related fungal phytoene desaturase encoding genes from *Neurospora crassa* (Schmidhauser et al., 1990), *Phycomyces* (Ruiz-Hidalgo et al., 1997), and *Cercospora nicotianae* (Ehrenshaft and Daub, 1994) contain respectively 2, 1, and 0 introns. Among these fungi the position of the introns in the phytoene desaturase encoding gene is not conserved as has been found with the fungal glyceraldehyde-3-phosphate dehydrogenase encoding genes (Verdoes et al., 1997).

Expression of the *X. dendrorhous* Phytoene Desaturase Encoding Gene in *E. coli*

For the conversion of phytoene into lycopene four enzymatic dehydrogenation reactions are required. Three different functional types of phytoene desaturase have been found. In cyanobacteria, algae, and higher plants two distinct enzymes are necessary for this four-step dehydrogenation process, the first phytoene desaturase (*crtP*-type) converts phytoene into zeta-carotene by two dehydrogenation reactions. This intermediate is further dehydrogenated into lycopene by a *zds*-type desaturase (*crtQ*). The phytoene desaturase of *Rhodobacter* carries out three desaturation steps yielding neurosporene. However, in most eubacteria and fungi these four steps are catalyzed by a single gene product encoded by a *crtI*-type gene (for reviews, see: Sandman, 1994; Armstrong and Hearst, 1996).

To determine the number of desaturase steps carried out by the phytoene desaturase protein of *X. dendrorhous* the cDNA clone (pPRpd1) was introduced in *E. coli* strain JM101 together with plasmid pACCRT-EB (Linden et al., 1991). This latter plasmid contains the genes encoding GGPP synthase (*crtE*) and phytoene synthase (*crtB*) from *E. uredovora* resulting in the accumulation of phytoene, the substrate for phytoene desaturase, in *E. coli*. The resulting transformant JM101[pACCRT-EB/pPRpd1] was red pigmented, and the carotenoid composition was analyzed by HPLC. It was shown that only all *trans*-lycopene was formed which exhibits the characteristic absorbance spectra at 445, 470, and 505 nm. These results indicate that *X. dendrorhous* contains a *crtI*-type phytoene desaturase, by which phytoene is dehydrogenated into lycopene.

Since accumulation of lycopene in *X. dendrorhous* was only observed in the presence of the inhibitor nicotine and since so far no lycopene accumulating strain of *X. dendrorhous* has been isolated, the question was raised whether lycopene was the substrate for the cyclization reactions (Girard et al., 1994). Based on the molecular genetic and biochemical evidence in *E. coli* we conclude that in *X. dendrorhous* lycopene is the end product of the desaturation steps and therefore is the most likely substrate for further cyclization reactions. Recently we isolated a *Xanthophyllomyces* gene (*crtB*+) which encodes a bifunctional protein involved in both phytoene synthesis from GGPP and cyclization of lycopene into β-carotene (Verdoes et al., manuscript in preparation). Apparently, mutations in the *crtB*+ gene will affect much more frequently phytoene synthesis than lycopene cyclization and therefore result in mutants which accumulate the colorless GGPP instead of the red lycopene.

For the fungus *Phycomyces blakesleeanus* (Bramley, 1985; Ruiz-Hidalgo et al., 1997) and the cyanobacterium *Synechococcus* (Sandmann and Kowalczyk, 1989), feed-

back regulation of phytoene desaturase has been demonstrated in vitro. This step may be the rate-limiting in astaxanthin biosynthesis in *X. dendrorhous* (Johnson and Schroeder, 1996) and therefore is one of the best targets to start metabolic pathway engineering. Presently, the effects of deregulation and overexpression of the *crtI* gene on astaxanthin biosynthesis in *X. dendrorhous* are being tested.

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ABBREVIATIONS

GGPP geranylgeranyl diphosphate
IPTG isopropyl-ß-D-thiogalactopyranoside

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