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1-Hydroxy monocyclic carotenoid 3,4-dehydrogenase from a marine bacterium that produces myxol

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Abstract A *crtD* (1-HO carotenoid 3,4-dehydrogenase gene) homolog from marine bacterium strain P99-3 included in the gene cluster for the biosynthesis of myxol (3′,4′-didehydro-1′,2′-dihydro-β,ψ-carotene-3,1′,2′-triol) was functionally identified. The P99-3 CrtD was phylogenetically distant from the other CrtDs. A catalytic feature was its high activity for the monocyclic carotenoid conversion: 1′-HO-torulene (3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-1′-ol) was prominently formed from 1′-HO-γ-carotene (1′,2′-dihydro-β,ψ-caroten-1′-ol) in *Escherichia coli* with P99-3 CrtD, indicating that this enzyme has been highly adapted to myxol biosynthesis. This unique type of *crtD* is a valuable tool for obtaining 1′-HO-3′,4′-didehydro monocyclic carotenoids in a heterologous carotenoid production system.

Keywords: 1-Hydroxycarotenoid 3,4-dehydrogenase; Substrate specificity; CrtD; 3′,4′-Didehydro-1′,2′-dihydro-β,ψ-caroten-1′-ol

1. Introduction

Certain species of bacteria, yeasts and fungi, as well as algae and higher plants synthesize a large number of carotenoids with different molecular structures. Most natural carotenoid diversity arises from differences in the type and level of desaturation and other modifications to the C40 backbone. Carotenoids have a variety of biological functions such as the stabilization of membrane fluidity, elimination of free radicals, photo protection, light harvesting, and as precursors for hormones [1]. Recent discoveries of their beneficial health-related properties have spurred great interest in the production of structurally diverse carotenoids for pharmaceutical applications [2].

Carotenoids are derived from the general isoprenoid (terpenoid) pathway. Tail–to-tail condensation of two molecules of geranylgeranyl pyrophosphate (GGPP) results in the formation of the first C40 carotenoid phytoene. This colorless carotenoid is converted to lycopene (ψ,ψ-carotene), which is a linear red-colored carotenoid, through four desaturation reactions via phytofluene, ζ-carotene and neurosporene. Cyclization of lycopene frequently follows carotene desaturation and precedes xanthophyll formation in the biosynthetic sequence. In anoxygenic photosynthetic bacteria, non-photosynthetic bacteria, and fungi, phytoene dehydrogenases (CrtI type) introduce three, four or five double bonds into phytoene consecutively, and yield neurosporene, lycopene, and 3,4-didehydrolycopene, respectively [3–5]. Phytoene dehydrogenases in cyanobacteria, algae and plants (Pds type) perform two dehydrogenations, generating ζ-carotene as the final product. ζ-Carotene dehydrogenase is subsequently required for generating lycopene in those organisms [3–5]. Dehydrogenases designated CrtD display amino acid sequence similarity to the CrtI-type phytoene dehydrogenases [6,7] and catalyze the introduction of C-3,4 double bonds into 1-hydroxy carotenoids [8,9]. The *crtD* genes have so far been cloned from purple photosynthetic bacteria, which form acyclic carotenoids [8–11]. The biosynthetic pathway just described is outlined in Fig. 1.

Marine bacterium strain P99-3 of the family *Flavobacteriaceae* produces myxol as the main carotenoid [13,14], which is a β-monocyclic carotenoid and is the aglycone of myxoxanthophyll commonly distributed in cyanobacteria [15]. The gene cluster for myxol synthesis has been cloned from strain P99-3 and a *crtD* homolog was identified in the cluster [13]. A *crtD* gene has never before been isolated from organisms which produce cyclic carotenoids. This led us to investigate the potential of the P99-3 homolog of CrtD for unique catalytic features, which would be beneficial for myxol synthesis and for the synthesis of diverse monocyclic carotenoids. This report describes the structural and functional analyses of the P99-3 CrtD.

2. Materials and methods

2.1. Bacterial strains, plasmids, oligonucleotides, and growth conditions

The bacterial strains, plasmids and oligonucleotides used in this study are listed in Table 1. *Escherichia coli* JM101 was grown on Luria–Bertani (LB) medium [21] at 28 °C. When required, the medium was supplemented with the following antibiotics at the indicated concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 20 μg/ml; tetracycline (Tc), 25 μg/ml; and kanamycin (Km), 25 μg/ml. IPTG (isopropyl β-D-thiogalactopyranoside) was added to the JM101 transformants containing pSO50, pSO53, or pUCZD to a final concentration of 0.1 mM when an optical density at 600 nm (OD_{600}) of 0.6 had been reached and growth was allowed to continue for 24 h more.

2.2. Genetic techniques

Standard recombinant DNA techniques were performed as described by Sambrook et al. [21].

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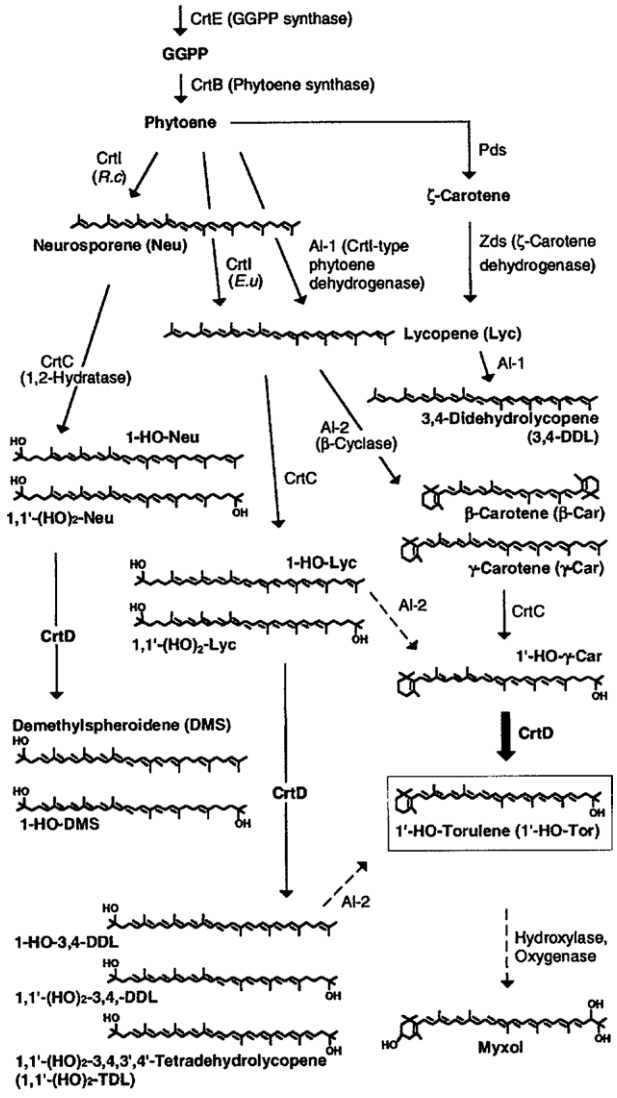

Fig. 1. Biosynthetic pathway to lycopene and subsequent cyclic and acyclic derivatives, and formation of substrate and product carotenoids involved in the reactions catalyzed by CrtD from strain P99-3 [9,12]. The dashed arrows indicate hypothetical reactions. The functions of the CrtD were experimentally confirmed in this study. *E.u* represents *Erwinia uredovora*; and *R.c*, *Rhodobacter capsulatus*.

2.3. Phylogenetic analysis

Homologous protein sequences in the non-redundant (NR) protein sequence database were retrieved by using version 2.0 of the BLAST program [22]. The protein sequences were aligned with ClustalW, version 1.7 [23], and a neighbor-joining tree was constructed by using the njplot program in this software. Gaps were included in the calculations.

2.4. In vivo complementation and analysis of carotenoid pigments

E. coli cells accumulating either a 1-HO-neurosporene, 1-HO-lycopene or 1′-HO-γ-carotene (1′,2′-dihydro-β,ψ-caroten-1′-ol) background were established by transformation with the appropriate plasmids (Table 1 and Fig. 2). These carotenoid-producing strains were then co-transformed with plasmid pUCZD. The resulting carotenoids were extracted with methanol containing 6% KOH from the cells of 50-ml cultures of the *E. coli* transformants by heating for 20 min at 60 °C and then partitioned in 50% diethyl ether in petrol. The carotenoid extracts were evaporated under nitrogen. HPLC analyses were carried out on either a Nucleosil C 18 column or Hypersil C 18 column at 20 °C, using acetonitrile/methanol/2-propanol (85:10:5, v/v/v) as the eluent. The separated carotenoids were detected with a Kontron 440 diode array detector and the resulting absorption spectra were recorded online. Authentic standards were used to identify the reaction products of CrtD by co-chromatography, these including various 3,4-desaturated derivatives generated in *E. coli* as indicated in Fig. 1.

3. Results and discussion

3.1. Characteristics of the deduced amino acid sequence

The deduced products of two open reading frames (ORFs) found in the gene cluster for the myxol synthesis of strain P99-3 showed homology to phytoene dehydrogenase (CrtI type) and less homology to 1-hydroxy carotenoid 3,4-dehydrogenase (CrtD) [13]. The product of one ORF, assigned as *crtI*, showed phytoene dehydrogenase activity, while that of the other ORF, designated *crtD*, did not show this activity [13]. The *crtD* gene encodes a protein of 488 amino acid residues [13] with a predicted molecular mass of 55 kDa. The characteristic amino acid sequence of ADP-binding βαβ-fold for FAD or NAD cofactors [24] is present at the N-terminus of CrtD (residues 3–31). The residues of an unknown function conserved at the C-termini of carotenoid dehydrogenases (CrtD and CrtI) from *Rba. capsulatus* [19] were also found at the C-terminus of the P99-3 CrtD (residues 455–470). Hydropathy analysis of the P99-3 CrtD [25] (http://www.bio.davidson.edu/courses/compbio/jems/project1page.html#References) did not reveal any putative transmembrane domains.

Table 1
Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristic(s)[a]	Reference/source
Strain		
E. coli JM101	Host strain for heterologous expression of *crt* genes	Toyobo
Plasmid		
pACCRT-EIB$_{Eu}$	Cm^r, lycopene-producing plasmid containing *crtEIB* from *E. uredovora*, p15A origin of replication	[16,17]
pACCRT-EBI$_{Rc}$	Cm^r, neurosporene-producing plasmid containing *crtEB* from *E. uredovora*, and *crtI* from *Rba. capsulatus*, p15A	[18]
pUCZD	Ap^r, *crtD* from strain P99-3 in pUC18, *lacZ*::*crtD*, pMB1 origin of replication	[13]
pRKcrtC	Tc^r, *crtC* from *Rba. capsulatus* in pRK404, RK2 origin of replication	[8]
pACCRT-EBIal2	Cm^r, γ-carotene-producing plasmid, lycopene cyclase gene *al-2* from *Neurospora crassa* in pACCRT-EIB$_{Eu}$	Unpublished plasmid; [12]
pFL104	Ap^r, *crtC* from *Rba. capsulatus* in pBR325	[19]
pSO50	Km^r, *crtDC* from *Rubrivivax gelatinosus* in pBBR1MCS-2, REP origin of replication	[20]
pSO53	Km^r, *crtD* from *Rvi. gelatinosus* in pBBR1MCS-2, REP	[9]

[a] Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant; Tc^r, tetracycline-resistant; Km^r, kanamycin-resistant.

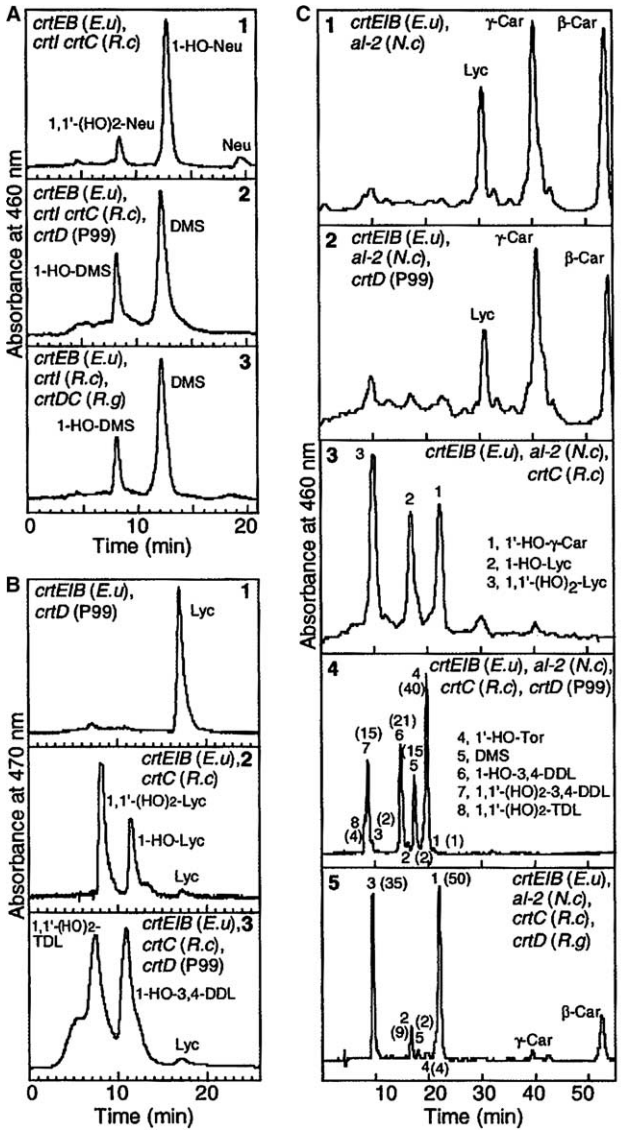

Fig. 2. HPLC elution profiles of the carotenoids produced in hydroxyneurosporene-accumulating *E. coli* (A), in lycopene- or hydroxylycopene-accumulating *E. coli* (B), and in γ-carotene or hydroxy-γ-carotene-accumulating *E. coli* (C) in the presence of P99-3 *crtD*. The carotenoid profiles of the hydroxyneurosporene- and hydroxy-γ-carotene-accumulating cells carrying *crtD* from *Rvi. gelatinosus* (*R.g*) are also shown (A3, C5). Genes on the plasmids introduced into *E. coli* and the carotenoids identified are indicated. *E.u* represents *E. uredovora*; *R.c*, *Rba. capsulatus*; P99, strain P99-3; and *N.c*, *N. crassa.* The amount of each hydroxy carotenoid is given as a percentage by weight of the total hydroxy carotenoids in parentheses next to the peak numbers (C4, C5). The plasmids contained in the *E. coli* cells were pACCRT-EBI$_{Rc}$ and pRKcrtC (A1); pACCRT-EBI$_{Rc}$, pRKcrtC and pUCZD (A2); pACCRT-EBI$_{Rc}$ and pSO50 (A3); pACCRT-EIB$_{Eu}$ and pUCZD (B1); pACCRT-EIB$_{Eu}$ and pRKcrtC (B2); pACCRT-EIB$_{Eu}$, pRKcrtC and pUCZD (B3); pACCRT-EBIal2 (C1); pACCRT-EBIal2 and pUCZD (C2); pACCRT-EBIal2 and pFL104 (C3); pACCRT-EBIal2, pRKcrtC and pUCZD (C4); and pACCRT-EBIal2, pRKcrtC and pSO53 (C5). Carotenoids were detected with the following absorbance maxima: 414, 442, 470 nm (Neu, 1-HO-Neu, 1,1′-(HO)$_2$-Neu); 425, 455, 485 nm (DMS, 1-HO-DMS); 440, 470, 505 nm (Lyc, 1-HO-Lyc, 1,1′-(HO)$_2$-Lyc); 450, 485, 518 nm (1-HO-3,4-DDL, 1,1′-(HO)$_2$-3,4-DDL); 465, 495, 530 nm (1,1′-(HO)$_2$-TDL); 430, 460, 490 nm (γ-Car, 1′-HO-γ-Car); 420, 455, 480 nm (β-Car); and 440, 475, 510 nm (1′-HO-Tor).

3.2. 1-Hydroxy carotenoid 3,4-dehydrogenase activity of P99-3 CrtD

To analyze the catalytic activity and determine suitable substrates to be efficiently converted, CrtD from strain P99-3 was expressed in *E. coli* transformants with different carotenoid backgrounds. The carotenoids formed were analyzed by high performance liquid chromatography (HPLC) (Fig. 2), and their HPLC retention times and UV–visible absorption spectra were compared with those of authentic carotenoid samples. CrtI from strain P99-3 introduces four double bonds into phytoene and yields lycopene [13], and therefore lycopene and γ-carotene were considered as potential substrates for the CrtD of P99-3 on the myxol biosynthetic pathway. However, lycopene and γ-carotene were not used as substrates by CrtD (B1 and C2). When *E. coli* produced 1-HO and 1,1′-(HO)$_2$-neurosporene (A1), both carotenoids were converted to the corresponding 3,4-dehydrogenated derivative by P99-3 CrtD (A2). The same result was observed for 1-HO and 1,1′-(HO)$_2$-lycopene (B3). All these reaction products were identified using the corresponding authentic carotenoids produced with the help of other CrtDs from photosynthetic bacteria (A3; data not shown for 1-HO-3,4-didehydrolycopene and 1,1′-(HO)$_2$-3,4,3′,4′-tetradehydrolycopene). 1-Hydroxy neurosporene or 1-hydroxy lycopene is the major substrate for the CrtDs from photosynthetic acyclic carotenoid-producing bacteria (Fig. 2A3) [8,9]. In the complementation with 1′-HO-γ-carotene and P99-3 *crtD*, 1′-HO-torulene (3′,4′-didehydro-1′, 2′-dihydro-β, ψ-caroten-1′-ol) (together with 1-HO-3, 4-didehydrolycopene and 1,1′-(HO)$_2$-3,4-didehydrolycopene from 1-HO- and 1,1′-(HO)$_2$-lycopene) accumulated (C4). This monocyclic carotenoid, found in *Spirochaeta aurantia* J1 as a major pigment [26], was identified by its spectral properties [26], a retention time slightly shorter than that of 1′-HO-γ-carotene and by the utilization of 1′-HO-γ-carotene upon introducing *crtD* into *E. coli* (C3, C4). These data clearly show that *crtD* from strain P99-3 encodes a 1-hydroxy carotenoid converting 3,4-dehydrogenase at the ψ-end group. The conversion reactions catalyzed by the P99-3 CrtD are indicated in Fig. 1.

Although neurosporene is an intermediate in the conversion of phytoene to lycopene by CrtI [13] and 1,1′-(HO)$_2$-lycopene is not an intermediate on the pathway to myxol, 1-hydroxy neurosporene and 1,1′-(HO)$_2$-lycopene were both efficient substrates for P99-3 CrtD (Fig. 2A2, B3). However, 1′-HO-γ-carotene and 1-HO-lycopene were the preferred substrates for the P99-3 CrtD (Fig. 2C4). These properties of this unique type of CrtD must reflect the cellular carotenoid biosynthetic pathway to the untypical monocyclic carotenoid myxol in strain P99-3 (Fig. 1).

3.3. P99-3 CrtD phylogenetically distant from the other CrtDs

To explore the phylogenetic relationships among P99-3 CrtD and the other CrtDs, an unrooted neighbor-joining tree was constructed based on the deduced amino acid sequences with the 7 most similar proteins, these all being CrtI-type phytoene dehydrogenases including 5 probable CrtI-type phytoene dehydrogenases, and with the 3 kinds of CrtI-type phytoene dehydrogenases, these being CrtI of *Rba. capsulatus* (three desaturations) [27], CrtI of *E. uredovora* (four desaturations)

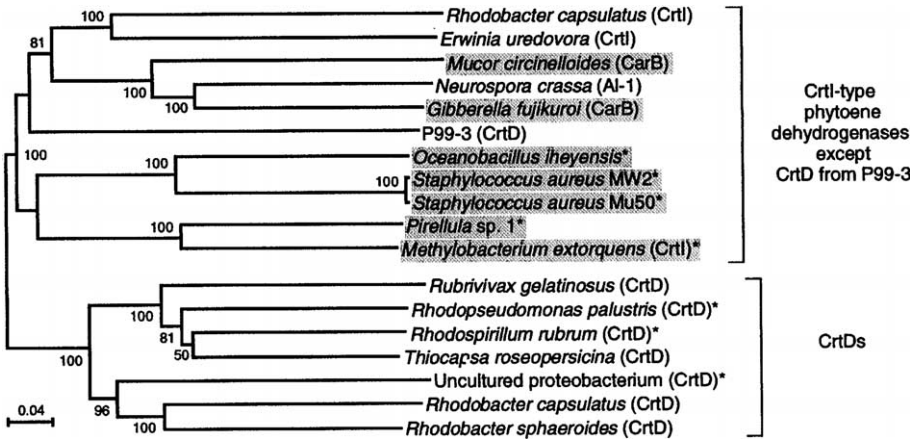

Fig. 3. Phylogenetic analysis of the P99-3 CrtD amino acid sequence with the 7 most similar proteins, all other CrtDs and the 3 kinds of CrtI type phytoene dehydrogenases. The 7 similar proteins (in gray) were retrieved from the NR protein sequence database. Proteins suspected to be CrtI-type phytoene dehydrogenase or CrtD (1-hydroxy carotenoid 3,4-dehydrogenase) are marked with an asterisk. All CrtDs available in the database are included. The number shown next to each node indicates the percentage bootstrap value of 100 replicates (only 50% or higher are cited). The scale bar indicates 0.04 substitution per amino acid site. The origins are as follows (GenBank numbers are shown in brackets): P99-3 [AB097813]; *Rba. capsulatus* (CrtI) [X52291]; *E. uredovora* [D90087]; fungus *N. crassa* [M57465]; fungus *Gibberella fujikuroi* [AJ426418] [30]; *Oceanobacillus iheyensis* [NC_004193]; *Staphylococcus aureus* MW2 [NC_003923]; *S. aureus* Mu50 [NC_002758]; fungus *Mucor circinelloides* [AJ238028] [7]; *Methylobacterium extorquens* [AY331188]; *Pirellula* sp. [NC_005027]; *Rba. capsulatus* (CrtD) [Z11165] [10]; *Rhodobacter sphaeroides* [AJ010302] [8]; *Rvi. gelatinosus* [AB034704] [9]; *Thiocapsa roseopersicina* [AF528191] [11]; *Rhodopseudomonas palustris* [BX572597]; *Rhodospirillum rubrum* [AY150801]; uncultured proteobacterium [AE008921].

[28] and Al-1 of *N. crassa* (up to five desaturations) [29] (Fig. 3). Two distinct clusters were apparent in the tree. One of these two clusters contained all CrtDs except the P99-3 CrtD. The P99-3 CrtD was included in the other cluster composed of CrtI-type phytoene dehydrogenases. This shows that P99-3 CrtD was evolutionarily distant from the other CrtDs. Strain P99-3 produces the β-monocyclic carotenoid, myxol, as the main carotenoid [14], while the bacteria from which all *crtDs* excepting P99-3 *crtD* are derived produce acyclic carotenoids [8–11].

3.4. Potential of the P99-3 CrtD for producing a variety of monocyclic carotenoids including 1′-HO-torulene

The catalytic properties of CrtDs from strain P99-3 and from *Rvi. gelatinosus* related to the synthesis of cyclic carotenoids were compared in 1′-HO-γ-carotene-producing *E. coli* (Fig. 2C4,C5). 1′-HO-Torulene constituted the main carotenoid (40% of the total hydroxylated carotenoids) in *E. coli* with P99-3 CrtD, while this carotenoid was a minor catalytic product (4%) of CrtD from *Rvi. gelatinosus*. These data indicate that, in contrast to CrtD from *Rvi. gelatinosus*, the P99-3 CrtD was highly adapted to dehydrogenate at C-3,4 of the 1-HO monocyclic carotenoid to achieve myxol biosynthesis, showing that a ß-ring at the other end of the molecule was tolerated by the P99-3 CrtD. Other CrtDs from *Rvi. gelatinosus* and from *Rba. sphaeroides* have been reported to exhibit only weak 3′,4′-dehydrogenase activity for 1′-HO-γ-carotene, and the CrtD from *Rvi. gelatinosus* has been reported not to show this activity for 1,1′-$(HO)_2$-lycopene in in vitro experiments [8,9]. Thus, P99-3 *crtD* must be the only 1-HO monocyclic carotenoid 3,4-dehydrogenase gene with the potential for the heterologous synthesis of a variety of monocyclic carotenoids in combination with other carotenogenic genes. Once 3,4-dehydrogenation has been catalyzed, oxygenation by CrtA [31] can be a subsequent reaction, and the β-ring may also be independently hydroxylated by CrtZ [28].

In conclusion, the 1-HO carotenoid 3,4-dehydrogenase CrtD from strain P99-3 was found to be phylogenetically distinct from the other CrtDs. The most prominent catalytic feature was its high activity for the monocyclic carotenoid conversion like 1′-HO-γ-carotene to 1′-HO-torulene. This makes P99-3 *crtD* gene a valuable tool for the heterologous production of 1′-HO-3′,4′-didehydro monocyclic carotenoids further substituted on the ß-ionone ring or on the acyclic part of the molecule.

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