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Structural and functional analysis of a lycopene β-monocyclase gene isolated from a unique marine bacterium that produces myxol

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Abstract **A gene coding for lycopene β-monocyclase, which metabolizes lycopene (ψ,ψ-carotene) to γ-carotene (β,ψ-carotene), was isolated for the first time from a unique marine bacterium strain P99-3 that produces myxol (a γ-carotene derivative). This lycopene β-monocyclase gene (designated *crtYm*) was included in the gene cluster which contained carotenoid biosynthetic gene (*crtI*, *crtB*, *crtZ*, *crtY*, and *crtA*) homologs. CrtYm, the CrtY homolog, metabolized lycopene to γ-carotene, which was confirmed by deletion/expression analysis of the *crtYm* and by subsequent analysis of the metabolites from lycopene based on the retention times on high-performance liquid chromatography, UV–visible absorption spectra, and mass spectrometry.**

Key words: Lycopene β-cyclase; γ-Carotene; Carotenoid; Marine bacterium; *Flavobacteriaceae*

1. Introduction

Certain species of bacteria, yeasts and fungi as well as algae and higher plants synthesize a great number of carotenoids of different molecular structures. Such carotenoids have a variety of biological functions, such as stabilization of membrane fluidity, elimination of free radicals, photo-protection, light harvesting, and as precursors for hormones [1]. Recently, these structurally diverse carotenoids have attracted greater attention due to their beneficial effects on human health, e.g. their potential in the prevention of diseases such as cancer and cardiovascular disease [2].

Carotenoids are derived from the general isoprenoid (terpenoid) pathway. Tail-to-tail condensation of two molecules of geranylgeranyl pyrophosphate 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. Cyclization of the lycopene is a pivotal branching point in the divergent carotenoid biosynthetic (crt) pathways. Synthesis of cyclic carotenoids involves the cyclization of one or both end groups of lycopene. Carotenoids with two β-ionone rings (β-rings) are ubiquitous, including β-carotene (β,β-carotene), zeaxanthin, canthaxanthin, and astaxanthin. Carotenoids with both β- and ε-rings are common in higher plants and contain α-carotene (β,ε-carotene) and lutein. A carotenoid having two ε-rings (ε,ε-carotene) is also present in lettuce, but is rare in nature. Myxoxanthophyll (myxol glycoside), which is a monocyclic carotenoid with one β-ring, is commonly distributed in cyanobacteria [3].

Genes encoding lycopene β-cyclases that introduce two β-rings into both ψ-end groups of lycopene to form β-carotene have been cloned from several bacteria and higher plants [4–11], while a gene(s) for lycopene β-monocyclase has not been reported. In higher plants, lycopene ε-cyclases usually synthesize monocyclic ε,ψ-carotene, with the exception of lettuce ε-cyclase that forms ε,ε-carotene. ε,ψ-Carotene formed is then thought to be converted to α-carotene by lycopene β-cyclase [9]. Genes for both the ε-monocyclase and the ε-bicyclase have been isolated more recently [9,12].

A marine bacterium, strain P99-3 (previously *Flavobacterium* sp.) [13], belonging to the family *Flavobacteriaceae* has recently been revealed to be a unique bacterium for which a new genus should be created (Fig. 1). Interestingly, this bacterium was found to produce myxol [13], which is the aglycone of myxoxanthophyll. Based on the molecular structure, myxol is thought to be synthesized by way of γ-carotene (β,ψ-carotene). It was therefore expected that this bacterium possesses a gene for lycopene β-monocyclase.

In the present study, we found a lycopene β-monocyclase gene in the *crt* gene cluster isolated from this strain P99-3. Our findings provide the molecular basis for lycopene β-monocyclase, which plays an important role in the synthesis of diverse monocyclic carotenoids having one β-ring.

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. The myxol-producing bacterial strain P99-3 (Marine Biotechnology Institute Culture collection number MBIC03313) was grown on Marine broth medium (Marine broth 2216; Difco) at 25°C. The *Escherichia coli* strains used were grown on Luria–Bertani (LB) medium [21] at 30°C. When required, the media were supplemented with the following antibiotics at the indicated concentrations: ampicillin (Ap), 100 μg/ml; and chloramphenicol (Cm), 20 μg/ml.

2.2. Genetic techniques

Plasmids were purified by QIAprep (Qiagen). Restriction endonuclease digestion and transformation of the *E. coli* strains were conducted by the methods of Sambrook et al. [21].

2.3. Preparation of the cosmid library

Total DNA was extracted from strain P99-3 as described previously

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[4], unless stated otherwise. Proteinase K was used instead of pronase E for protease treatment. NaCl was added at a final concentration ranging from 0.1 to 0.2 M to DNA solution after phenol–chloroform extraction.

The total DNA from strain P99-3 was partially digested with *Sau*3AI and ligated into the *Bam*HI site of SuperCos 1. The ligates were packaged into lambda bacteriophage by using packaging extracts (LAMBDA INN; Wako, Osaka, Japan). *E. coli* XL1-Blue MR transformed with pACCRT-EIB was infected with the resultant phages, and 1000 library clones resistant to both Ap and Cm were isolated.

2.4. Nucleotide sequencing and computer analysis

In order to determine the nucleotide sequence, subfragments digested with appropriate restriction endonucleases were cloned into the multiple cloning site of pBluescript II KS–. The nucleotide sequences were determined in both orientations by means of a DNA sequencing kit (Dye Terminator Cycle Sequence; Perkin-Elmer) and a model 3700 DNA sequencer (Perkin-Elmer) according to the manufacturer's instructions. The DNA sequences were assembled using the Sequencher, version 3.0, (Gene Codes Corporation). Homologous protein sequences in the protein sequence database were retrieved using version 2.0 of the BLAST program [22]. The protein sequences were aligned using Clustal W, version 1.7 [15]. Gaps were excluded from the calculations.

2.5. Analysis of carotenoid pigments accumulated in E. coli

E. coli XL1-Blue MR carrying both pACCRT-EIB and each SuperCos 1 derivative that includes an inserted P99-3 DNA fragment was grown for 2 days, while *E. coli* JM109 carrying both pACCRT-EIB and each pBluescript derivative that includes an inserted *crt* gene(s) was grown overnight. The *E. coli* cells were then harvested by centrifugation, and were shaken vigorously for 30 min after the addition of a volume of acetone sufficient to extract carotenoid pigments. The extracts were centrifuged at 14 000 × *g* for 5 min at 4°C in order to remove cell debris, and the resulting supernatants were subjected to high-performance liquid chromatography (HPLC), or used for purification through several chromatographic steps, as described below.

The liquid phase obtained was put through HPLC analysis at 25°C on an octadecyl silica reverse-phase column (3.9 by 300 mm; Novapak HR 6 μm C_{18}; Waters) using a photodiode array detector (Waters model 2996). It was developed at a flow rate of 1 ml/min with acetonitrile–methanol–2-propanol [45:3:2 (vol/vol/vol)], and monitored at maximal absorbance between 250 and 600 nm.

The liquid phase obtained by the procedure described above was concentrated in vacuo, and extracted using chloroform–methanol (3:1). The organic layer was concentrated in vacuo, and loaded onto a column of silica-gel 60 (Merck). The pigments were eluted with *n*-hexane. The carotenoids were then collected from HPLC as described above.

Relative molecular masses of the purified carotenoids were measured by field-desorption mass spectrometry using a double-focusing gas chromatograph/mass spectrometer equipped with a field-desorption apparatus (M-2500; Hitachi, Japan) [23].

2.6. Nucleotide sequence accession number

The nucleotide sequence of the 8.9-kb *Bgl*II–*Sal*I region has been

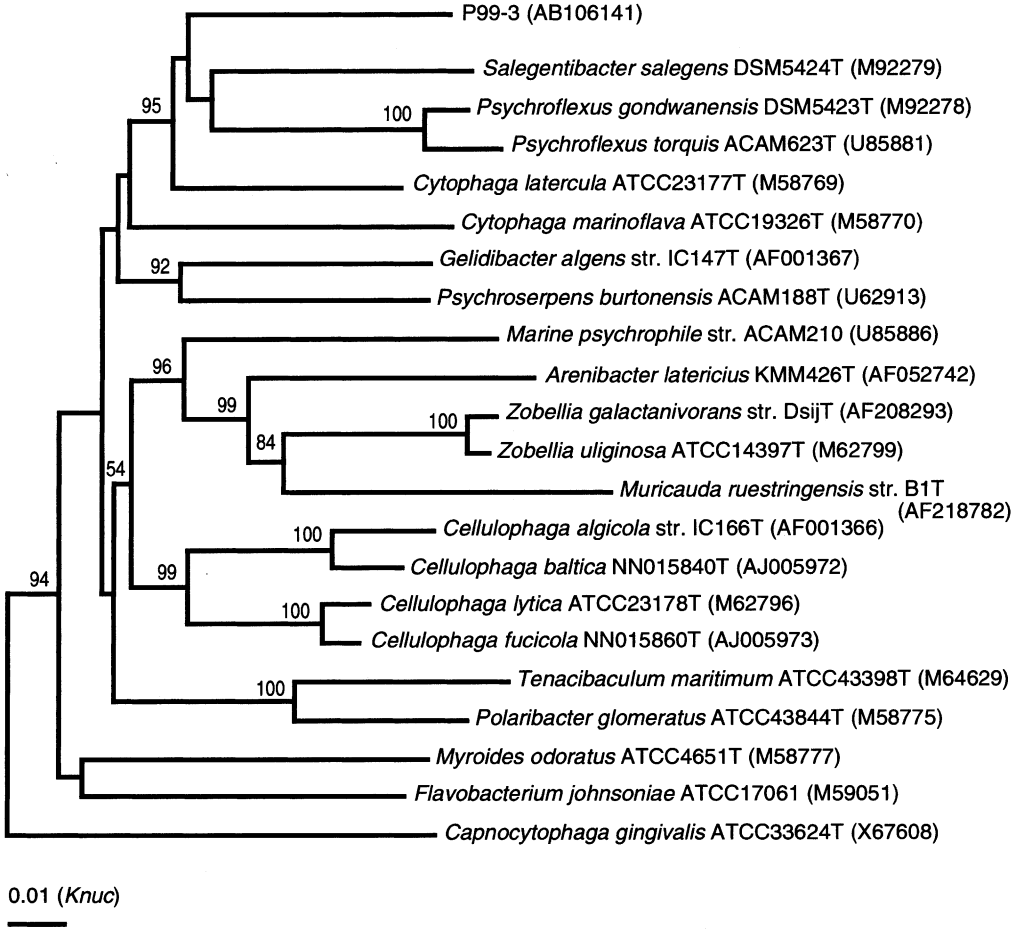

Fig. 1. Phylogenetic position of a myxol-producing bacterium P99-3 (previously *Flavobacterium* sp.) [13] deduced from 16S rDNA sequences (Makoto Suzuki and Hiroaki Kasai, personal communication). The determined sequence of the P99-3 was aligned to the alignment based on the secondary structure model which is maintained by the SSU rRNA database [14] using the software program Clustal W [15]. The evolutionary distances were then computed with the DNADIST program in the PHYLIP package [16] with the Kimura '2-parameter' model [17], and the phylogenetic tree was constructed by using the neighbor-joining method [18]. The number shown next to each node indicates the percentage bootstrap value of 100 replicates (only 50% or higher were cited). The scale bar indicates a genetic distance of 0.01 (*Knuc*) [17].

Table 1
Bacterial strains, plasmids, and oligonucleotides used in this study

Strain/plasmid[a]	Relevant characteristic(s)[b]	Reference/ source
Strains		
Strain P99-3	Myxol-producing bacterium	[13]
E. coli XL1-Blue MR	Host strain of cosmid vector, SuperCos 1 and for heterologous expression of *crt* genes	Stratagene
E. coli DH5α	Host strain for DNA manipulation	Toyobo
E. coli JM109	Host strain for DNA manipulation and for heterologous expression of *crt* genes	Toyobo
Plasmids		
pACCRT-EIB	Cm^r, lycopene producing plasmid containing *crtEIB* from *E. uredovora*, p15a origin of replication	[19]
pACCRT-EB	Cm^r, phytoene producing plasmid carrying *crtEB* from *E. uredovora*, p15a	[8]
pACCAR16ΔcrtX	Cm^r, β-carotene producing plasmid containing *crtEYIB* from *E. uredovora*, p15a	[20]
SuperCos 1	Ap^r, cosmid vector	Stratagene
pBluescript II KS–	Ap^r, cloning vector	Toyobo
pBluescript SK–	Ap^r, cloning vector	Toyobo
pUC18	Ap^r, cloning vector	Takara
pSC601	Ap^r, ca. 40-kb fragment (partial *Sau*3AI digest) of strain P99-3 DNA cloned in the *Bam*HI site of SuperCos 1	This study
pBS603	Ap^r, 4.7-kb *Sal*I–*Sac*I fragment of pSC601 cloned into pBluescript II KS–	This study
pBS606	Ap^r, 8.9-kb *Bgl*II–*Sal*I fragment of pSC601 in pBluescript SK–	This study
pBS603dD	Ap^r, pBS603 digested with *Eco*RV and religated, resulting in the elimination of 0.7-kb *Eco*RV fragment within *crtD*	This study
pBS603dDA	Ap^r, pBS603dD digested with *Nde*I and religated, resulting in the elimination of 0.2-kb *Nde*I fragment within *crtA*	This study
pBS603dDO	Ap^r, pBS603 digested with *Eco*RV and *Eco*RI and religated with insertion of an *Eco*RI–*Not*I–*Bam*HI adapter (Takara), resulting in the elimination of 1.5-kb *Eco*RV–*Eco*RI fragment within the region containing *crtD* and *orf1*	This study
pBS603dDOYm	Ap^r, pBS603 digested with *Eco*RV and *Nru*I and religated, resulting in the elimination of 2.3-kb *Eco*RV–*Nru*I fragment within the region containing *crtD*, *orf1* and *crtYm*	This study
pBS603dDOA	Ap^r, pBS603dDO digested with *Nde*I and religated, resulting in the elimination of 0.2-kb *Nde*I fragment within *crtA*	This study
pBSY	Ap^r, 1.9-kb *Hin*dIII–*Bam*HI fragment of pACCAR16ΔcrtX containing *crtY* cloned into pBluescript SK–	This study
pUCZI	Ap^r, *Eco*RI-*Sal*I-cleaved PCR fragment carrying *crtI* amplified with I-EI-Fw and I-SalI-Rv primers from pSC601 cloned into pUC18, *lacZ*::*crtI*	This study
pUCZD	Ap^r, *Eco*RI–*Bgl*II-cleaved PCR fragment carrying *crtD* amplified with D-EI-Fw and D-BglII-Rv primers from pSC601 cloned into pUC18, *lacZ*::*crtD*	This study
Oligonucleotides		
I-EI-Fw	TACGAATTCGATGAATAAAAAGATAGCAATTATAGG	
I-SalI-Rv	CAGGTCGACTTATAGCGAGTATTTTTTAACCAAC	
D-EI-Fw	TACGAATTCGATGAAAAAGGCTATCATTATAGGG	
D-BglII-Rv	CATAGATCTCCCATAGCATGATGATAGTAA	

[a]Plasmids pACCRT-EIB, pACCRT-EB, and pACCAR16ΔcrtX are constructed using the *E. coli* vector pACYC184.
[b]Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant.

Table 2
The *crt* gene products of strain P99-3 and homology with other proteins

Product (Residues)	Possible function	Identity with other gene products (%)[a]
Orf2 (257)	Unknown	
CrtI (487)	Phytoene desaturase	CarB (30), CarA2 (29), CrtI of *Erwinia* (26), CrtI of *Paracoccus* (25), CrtD (25)
CrtB (279)	Phytoene synthase	CrtB of *Streptomyces* (31), CrtB of *Spirulina* (27), CrtB of *Paracoccus* (27), Psy (26), CrtB of *Erwinia* (25), Al-2 (25)
CrtZ (148)	γ-Carotene β-ring hydroxylase	CrtZ of *Erwinia* (39), CrtZ of *Paracoccus* (32)
CrtD (488)	γ-Carotene 3′,4′-desaturase	CarB (29), CrtI of *Erwinia* (27), CarA2 (27), CrtI of *Paracoccus* (27), CrtD (26)
Orf1 (207)	Unknown	
CrtYm (377)	Lycopene β-monocyclase	CrtL-1 (24), CrtY of *Paracoccus* (23), CrtY of *Erwinia* (21)
CrtA (239)	Monooxygenase	CrtA (29)

[a]Percentage identity at the amino acid level is given: CarB is from fungus *Phycomyces blakesleeanus* (DDBJ accession number P54982); CarA2, *Myxococcus xanthus* DK1050 (P54979) [25]; CrtI, CrtB, CrtZ and CrtY of *Erwinia*, *E. uredovora* 20D3 (GenBank no. D90087) [4]; CrtI, CrtB, CrtZ and CrtY of *Paracoccus*, *Paracoccus* sp. MBIC01143 (previously called *Agrobacterium aurantiacum*) (GenBank no. D58420) [8]; CrtD, *Rhodobacter sphaeroides* ATCC 17023 (Q01671) [26,27]; CrtB of *Streptomyces*, *Streptomyces griseus* JA3933 (P54977) [28]; CrtB of *Spirulina*, Cyanobacterium *Spirulina platensis* IAM M-135 (DDBJ accession number O07333); Psy, bell pepper (P37272) [29]; Al-2, fungus *Neurospora crassa* (P37295) [30]; CrtL-1, tobacco (X81787) [10]; CrtA, *Rhodobacter capsulatus* SB1003 (P17055) [31].

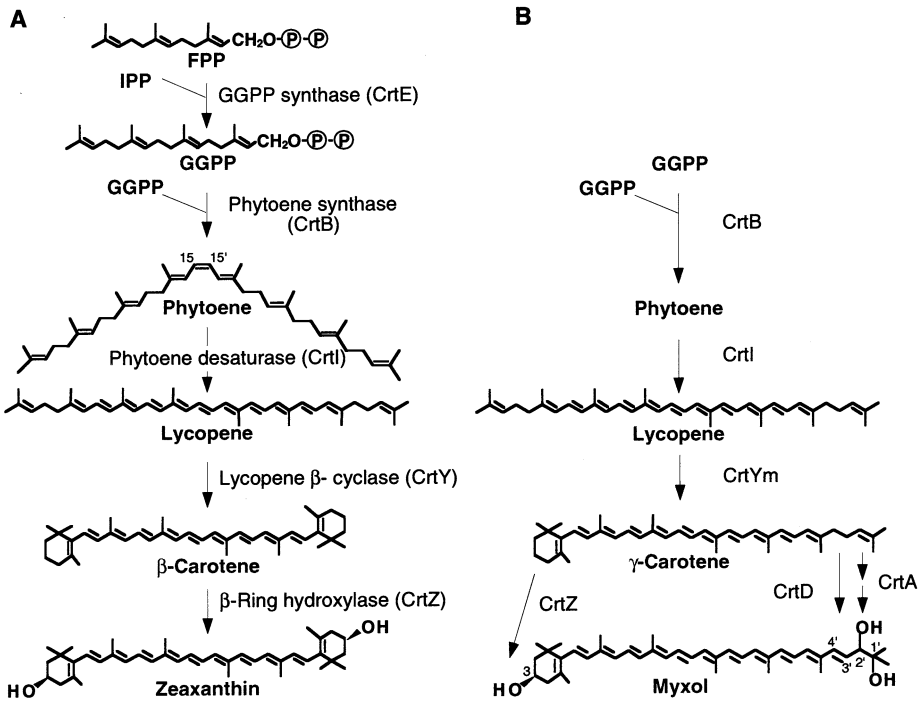

Fig. 2. Carotenoid biosynthetic pathway to zeaxanthin for *E. uredovora* 20D3 and the functions of individual carotenoid biosynthetic enzymes encoded on the *E. uredovora crt* gene cluster [4,8] (A) and a possible myxol biosynthetic pathway for the marine bacterium strain P99-3 and feasible functions of individual carotenoid biosynthetic enzymes encoded on the P99-3 *crt* gene cluster (B). Functions of CrtI and CrtYm of P99-3 were experimentally confirmed, while those of the other Crt products of P99-3 were suggested by their deduced amino acid sequence homologies. FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate.

deposited in the DDBJ/EMBL/GenBank database under accession no. AB097813.

3. Results and discussion

3.1. Cloning of a lycopene β-monocyclase gene

The *E. coli* strain carrying plasmid pACCRT-EIB accumulates lycopene due to the expression of the *crtE*, *crtB*, and *crtI* genes (Fig. 2A), which are derived from the eubacterium *Erwinia uredovora* 20D3 [8]. In order to clone the lycopene β-monocyclase gene, a cosmid DNA library of the myxol-producing marine bacterium strain P99-3 was constructed using *E. coli* strain XL1-Blue MR carrying pACCRT-EIB.

The formation of a β-monocyclic product, γ-carotene, from lycopene by lycopene β-monocyclase was considered to result in a change of color from red to red-yellow. Thus, 300 colonies that seemed to turn yellowish on plates were picked up from the 1000 library colonies. HPLC analysis was then performed for a second screening of these 300 clones. Because lycopene and β-carotene were eluted at ca. 20 and ca. 40 min on HPLC, respectively, γ-carotene should be eluted between 20 and 40 min. As a result, three clones out of the 300 were found to synthesize a pigment with a retention time of ca. 30 min. The visible absorption spectrum of this pigment with absorption maxima at 464 and 492 nm closely resembled that of authentic γ-carotene reported in the litera-

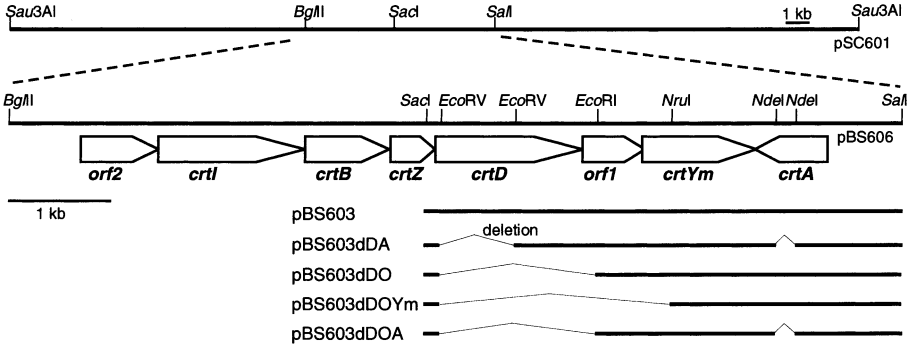

Fig. 3. Genetic organization of the *crt* gene cluster of the strain P99-3. Open boxes indicate the size and direction of the ORFs identified. Thick lines with names of plasmids indicate the DNA fragments derived from strain P99-3. Plasmids pBS606 and pBS603 contain subfragments from pSC601. pBS603dDA, pBS603dDO, pBS603dDOYm, and pBS603dDOA are deletion derivatives from pBS603. pBS603dDA contains *orf1* and *crtYm*. pBS603dDO contains *crtYm* and *crtA*. pBS603dDOYm contains *crtA* alone, while pBS603dDOA contains *crtYm* alone. In these pBS603 derivatives as well as pBS603, the orientation of the *lac* promoter of the vector pBluescript II KS– is the same as that of *crtA*.

ture [24]. A plasmid isolated from one of the three positive clones was named pSC601, and used for further experiments.

3.2. Identification of a crt gene cluster including the lycopene β-monocyclase gene

The pSC601 was found to contain a ca. 40-kb DNA fragment from strain P99-3 (Fig. 3), and the fragment inserted was subcloned into pBluescript vector. One of the resulting plasmids, named pBS603, which contained a 4.7-kb *Sal*I–*Sac*I fragment from pSC601 (Fig. 3), allowed the lycopene-accumulating *E. coli* carrying pACCRT-EIB to synthesize a small amount of the pigment with a retention time of ca. 30 min on HPLC (Fig. 4A). The nucleotide sequence of the 4.7-kb *Sal*I–*Sac*I fragment revealed four open reading frames (ORFs) as shown in Fig. 3. Three ORFs were similar to the known *crt* genes, *crtD* (*crtI*), *crtY*, and *crtA*. Thus, the four ORFs were designated *crtD*, *orf1*, *crtYm*, and *crtA* (Fig. 3 and Table 2). The deduced product of *crtYm*, with a predicted molecular mass of 43 kDa, was most similar to lycopene β-cyclase (bicyclase) of tobacco (*Nicotiana tabacum*) (24%) or to that of *Paracoccus* sp. MBIC01143 (23%) (Table 2). These results strongly suggest that the *crtYm* gene encodes lycopene β-monocyclase. The nucleotide sequence of the DNA region upstream of *crtD* was also determined, and four ORFs were found (Fig. 3). Three ORFs were similar to the known *crt* genes, *crtI* (*crtD*), *crtB*, and *crtZ*. Thus, the four ORFs found were designated *orf2*, *crtI*, *crtB*, and *crtZ* (Fig. 3 and Table 2). The translated sequences of *orf1* and *orf2* showed no significant homology with any other proteins. However, the deduced product of *orf1* was most similar to glutathione reductase in rice (18% identity) (GenBank accession no. P48642) [32]. No ORF was found upstream of *orf2* nor downstream of *crtA* on pBS606 (Fig. 3).

The deduced amino acid sequences of *crtI* as well as *crtD* appearing in the cluster showed similarities to those of both phytoene and hydroxyneurosporene (C-3′,4′) desaturases to similar degrees (Table 2). The *crtI* was introduced in frame at the 5′ terminus of *lacZ* on pUC18 vector. The resulting plasmid pUCZI was found to allow the phytoene-accumulating *E. coli* containing pACCRT-EB to produce lycopene (data not shown), indicating that *crtI* encodes phytoene desaturase. On the other hand, a pUC18 derivative containing *crtD* at the 5′ terminus of *lacZ* on the vector in frame (named pUCZD) did not allow the phytoene-accumulating *E. coli* to produce lycopene (data not shown), suggesting that *crtD* codes for C-3′,4′ desaturase that dehydrogenates at C-3′,4′ of γ-carotene toward the synthesis of myxol, a main carotenoid produced by the strain P99-3 [13] (Fig. 2B). CrtZ was thought to hydroxylate at C-3 on the β-ring of γ-carotene. CrtA is likely to be involved in additional hydroxylation at positions C-2′ and/or perhaps C-1′ of γ-carotene.

Myxol could not be detected from the cells of *E. coli* that contained both pBS606 (Fig. 3) and pACCRT-EIB. Lycopene and the pigment with a retention time of ca. 30 min on HPLC were the only pigments that could be detected from the *E. coli* cells containing both pBS606 and pACCRT-EIB (data not shown). However, we cannot conclude that the genes on pBS606 were insufficient for the synthesis of myxol in strain P99-3. The activity of CrtI could be detected with pUCZI, but not with pBS603 in *E. coli*. Therefore, it is possible that other genes on pBS606 were also not expressed in *E. coli* due to the difference in expression mechanisms between *E. coli* and strain P99-3.

3.3. Functional identification of CrtYm as lycopene β-monocyclase and inhibition of CrtYm activity by the coexistence of orf1

Deletion derivatives of pBS603 were constructed (Table 1 and Fig. 3), and introduced into the lycopene-accumulating *E. coli* strain carrying pACCRT-EIB. Carotenoid pigments extracted from the individual *E. coli* cells were analyzed by HPLC (Fig. 4). Deletions of *crtD* and *crtA* had no apparent effect on the pigment compositions of the cells (Fig. 4B). On the other hand, a marked decrease in the lycopene content and the simultaneous increase in the pigment content thought to be γ-carotene were observed with the cells with a deletion of *orf1* (pBS603dDO) (Fig. 4C).

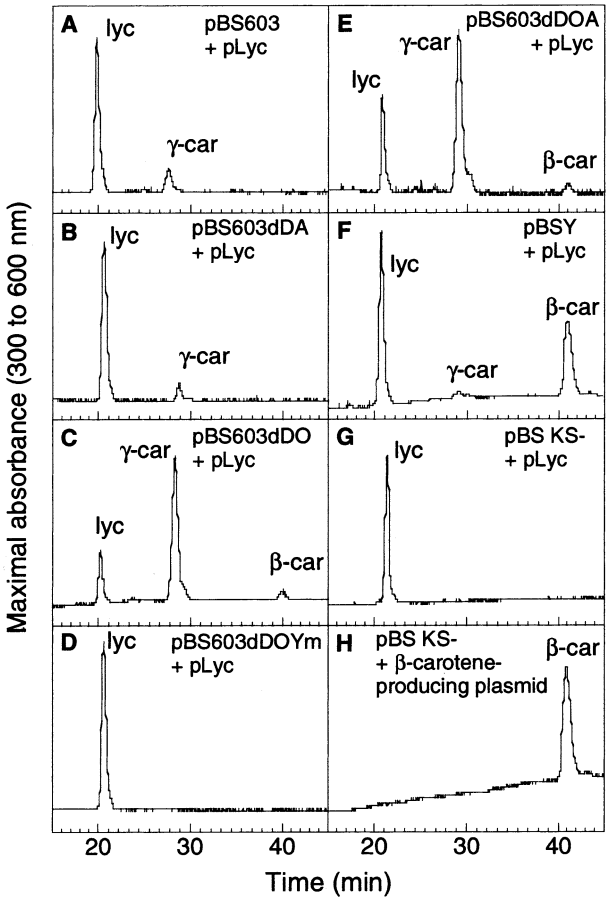

Fig. 4. HPLC elution profiles of carotenoids produced in the lycopene-accumulating *E. coli* cells carrying pACCRT-EIB in the presence of the *crt* gene(s) of strain P99-3 or the *crtY* gene of *E. uredovora* 20D3. Two plasmids contained in *E. coli* are indicated. pBS603dDA, pBS603dDO, pBS603dDOYm, and pBS603dDOA are the deletion derivatives of pBS603, and structural information of these plasmids is given in Fig. 3. pBSY contains the *crtY* gene of *E. uredovora* 20D3 in pBluescript SK–. This *crtY* was cloned in a divergent organization relative to the *lac* promoter on pBluescript vector, because *crtYm* was also cloned in this manner (pBS603dDOA). pACCRT-EIB, here indicated as pLyc, is the lycopene-producing plasmid. The carotenoid profiles of the cells carrying two control plasmids pBluescript II KS– (here indicated as pBS KS–) and pACCRT-EIB (G) and of the cells carrying pBluescript II KS– and a β-carotene-producing plasmid (pACCAR16ΔcrtX) (H) are also shown. lyc, lycopene; γ-car, γ-carotene; β-car, β-carotene.

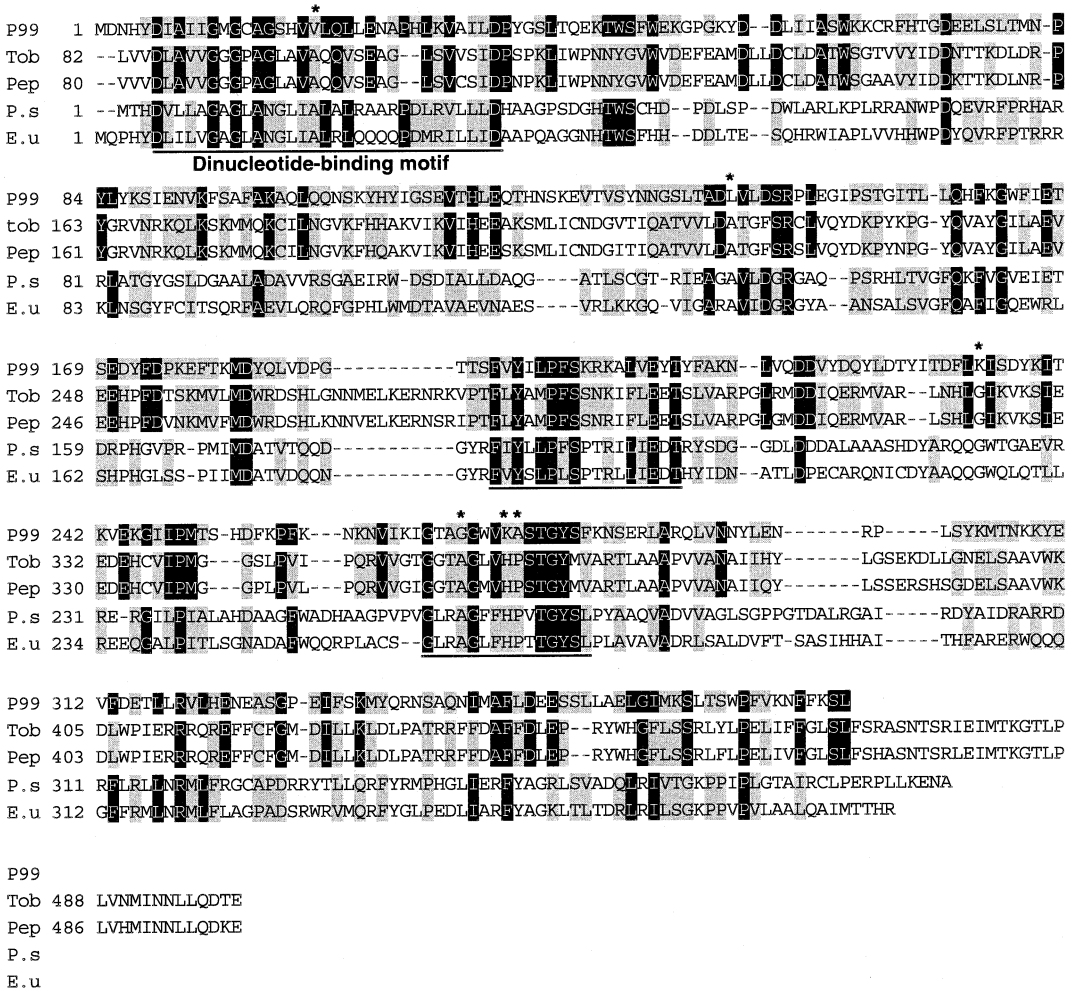

Fig. 5. Alignment of deduced amino acid sequences of CrtYm (from strain P99-3) and lycopene β-cyclases from higher plants (Tob and Pep) and other bacteria (P.s and E.u). Identical residues and similar residues are shown in black and in gray, respectively, for CrtYm and β-cyclases from higher plants or from bacteria. Three conserved regions are underlined. Asterisks indicate residues identical for all of the aligned β-cyclases except CrtYm. Numbers denote the number of the amino acid residue that starts the row. Sequence alignment was obtained through the use of the software program Clustal W [15]. Gaps introduced to maximize the alignment are dashed. The origins of the aligned β-cyclases are as follows: P99, CrtYm from a marine bacterium strain P99-3 (GenBank no. AB097813) (this study); Tob, CrtL-1 from tobacco (GenBank no. X81787) [10]; Pep, Lcy1 from bell pepper (GenBank no. Q43415) [7]; P.s, CrtY from *Paracoccus* sp. MBIC01143 (GenBank no. D58420) [8]; E.u, CrtY from *E. uredovora* 20D3 (GenBank no. D90087) [4]. All β-cyclases except CrtYm are lycopene β-bicyclases. Amino acid residues for transit peptides of higher plant enzymes are not shown.

This pigment was extracted from the *E. coli* cells containing both the pACCRT-EIB and pBS603dDO, and purified. The relative molecular mass of this pigment was 536. Together with its HPLC retention time and UV–visible absorption spectrum, this pigment was confirmed to be γ-carotene.

Cells with a deletion of *crtYm* showed impaired ability to accumulate γ-carotene (Fig. 4D). The introduction of *crtYm* alone (pBS603dDOA) into the lycopene-accumulating *E. coli* decreased the amount of lycopene accumulated in the cells and yielded almost exclusively γ-carotene. As a byproduct, β-carotene was barely detectable (Fig. 4E). These results demonstrate that the *crtYm* encodes lycopene β-monocyclase. In contrast, the introduction of the *crtY* gene derived from the eubacterium *E. uredovora* 20D3 (pBSY), which encodes lycopene β-cyclase, into lycopene-accumulating *E. coli* carrying pACCRT-EIB resulted almost exclusively in the synthesis of bicyclic carotenoid β-carotene from lycopene (Fig. 4F). This result is consistent with the findings that the lycopene β-cyclases so far characterized are lycopene β-bicyclases [4–11]. CrtYm may have a much weaker affinity than the other lycopene β-cyclases for γ-carotene as the substrate.

Deletion of *orf1* on pBS603dDA resulted in an increased yield of γ-carotene (Fig. 4B,E), suggesting that Orf1 might have markedly inhibited CrtYm activity. The *orf1* gene was immediately upstream of *crtYm* in a head-to-tail orientation and overlapped *crtYm* by 22 bp (Fig. 3). It is conceivable that the strain P99-3 involves a mechanism to overcome the *orf1*-mediated inhibition, since this strain produced myxol as the main carotenoid product. Elucidation of the *orf1*-mediated unknown mechanism may reveal the important molecular machinery for the expression of the myxol synthetic pathway in strain P99-3.

3.4. Comparison of the deduced amino acid sequences of CrtYm and other lycopene β-cyclases

An alignment of the amino acid sequences of CrtYm and other β-cyclases from higher plants and bacteria was generated (Fig. 5). Three conserved regions were found. One out

of the three regions contains a dinucleotide-binding motif [33]. Purified lycopene β-cyclase from *E. uredovora* 20D3 required reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for its cyclase activity, and this region was suggested to be involved in the binding of NADH and NADPH [34]. Other cyclases including CrtYm may also require such a dinucleotide for the activity. The three regions were also conserved in lycopene ε-cyclases from *Arabidopsis thaliana* and tomato (monocyclases) and from lettuce (bicyclase) (data not shown). Consequently, the two other conserved regions were suggested to be important for the cyclization reaction or lycopene recognition. Six amino acid residues were identified which were conserved in all the aligned β-cyclases except CrtYm. It is possible that at least one of the six amino acid residues may determine the number of β-rings added to lycopene, because a single amino acid residue of lycopene ε-cyclase was shown to determine the number of ε-rings formed (one ring or two) [12]. Such an amino acid residue of β-cyclase remains to be clarified. Our discovery of the lycopene β-monocyclase gene sheds light on the molecular basis of the synthesis of diverse monocyclic carotenoids having one β-ring, and will facilitate the investigation of their therapeutic effects.

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