A novel carotenoid 1,2-hydratase (CruF) from two species of the non-photosynthetic bacterium *Deinococcus*

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A novel carotenoid 1,2-hydratase (CruF) responsible for the C-1',2' hydration of γ -carotene was identified in the non-photosynthetic bacteria *Deinococcus radiodurans* R1 and *Deinococcus geothermalis* DSM 11300. Gene expression and disruption experiments demonstrated that *dr0091* and *dgeo2309* encode CruF in *D. radiodurans* and *D. geothermalis*, respectively. Their homologues were also found in the genomes of cyanobacteria, and exhibited little homology to the hydroxyneurosporene synthase (CrtC) proteins found mainly in photosynthetic bacteria. Phylogenetic analysis showed that CruF homologues form a separate family, which is evolutionarily distant from the known CrtC family.

INTRODUCTION

Carotenoids, natural antioxidative pigments important to human health, are usually synthesized in bacteria, algae, fungi and plants, and typically consist of C₄₀ hydrocarbon backbones (carotenes) and their oxygenated derivatives (xanthophylls) (Armstrong, 1997). Biosynthesis of C₄₀ carotenoid begins at the isoprenoid or terpenoid pathway. The head-to-head condensation of two geranylgeranyl diphosphate (GGPP) molecules forms a colourless carotenoid phytoene, catalysed by phytoene synthase (CrtB). Phytoene is then converted to lycopene by phytoene desaturase (CrtI), or by phytoene desaturase (CrtP), ζ -carotene desaturase (CrtQ) and *cis*-carotene isomerase (CrtH) (Takaichi & Mochimaru, 2007). Following desaturation, carotenoid biosynthesis is diversified into acyclic and cyclic carotenoids. For cyclization, one or both ψ -end groups of lycopene are cyclized into γ -carotene or β -carotene by lycopene cyclase. For further modification of acyclic and monocyclic carotenoids, the ψ -end group can be hydrated by carotenoid 1,2-hydratase at C-1 (or 1') and C-2 (or 2') (Schmidt-Dannert, 2000).

Species of the genus *Deinococcus* are red-pigmented non-photosynthetic bacteria, well known for their extreme tolerance to ionizing radiation and numerous oxidative

damaging agents (Cox & Battista, 2005; Ferreira *et al.*, 1997). *D. radiodurans* produces deinoxanthin, a unique hydroxylated carotenoid, as its major carotenoid (Lemee *et al.*, 1997; Saito *et al.*, 1998). Deinoxanthin was shown to have stronger reactive oxygen species (ROS) scavenging ability than lycopene and β -carotene, due to its extended conjugated double bonds and functional end groups, such as hydroxyl groups (Tian *et al.*, 2007). We have previously identified phytoene synthase (CrtB, DR0862), phytoene desaturase (CrtI, DR0861) and carotenoid 3',4'-desaturase (CrtD, DR2250), which are involved in steps of deinoxanthin biosynthesis (Tian *et al.*, 2007, 2008; Xu *et al.*, 2007). Zhang *et al.* (2007) also confirmed the functions of the *crtB* and *crtI* genes. Two other genes, encoding carotenoid ketolase (CrtO) and lycopene cyclase (CrtLm), in *D. radiodurans* have been described (Tao & Cheng, 2004; Tao *et al.*, 2004). Carotenoid 1,2-hydratase, a carotenoid biosynthetic enzyme, is required to catalyse the synthesis of deinoxanthin by hydration at the C-1',2' double bond. CrtC-type carotenoid 1,2-hydratases have been found in photosynthetic bacteria (Armstrong *et al.*, 1989; Frigaard *et al.*, 2004; Kovács *et al.*, 2003; Ouchane *et al.*, 1997), primarily involved in the biosynthesis of spirilloxanthin, spheroidene, chlorobactene and its derivatives. However, no homologue of the CrtC-type carotenoid 1,2-hydratase has been detected in the *D. radiodurans* genome (Makarova *et al.*, 2001; White *et al.*, 1999). We identified the gene candidates of carotenoid 1,2-hydratase, *dr0091* and *dgeo2309*, predicted in the gene neighbourhood of *crtO*

Abbreviations: GGPP, geranylgeranyl diphosphate; ROS, reactive oxygen species.

A supplementary table of primers and two supplementary figures are available with the online version of this paper.

from *D. radiodurans* R1 and *D. geothermalis* DSM 11300, respectively. The homologue of *dr0091* and *dgeo2309* from the cyanobacterium *Synechococcus* sp. PCC 7002 was recently described and named CruF by Maresca *et al.* (2008).

In this study, we identified and characterized a CruF-type carotenoid 1,2-hydratase from *D. radiodurans* R1 and *D. geothermalis* DSM 11300. The results helped to reveal the biosynthetic pathway of deinoxanthin in *D. radiodurans* (Fig. 1). The CruF homologues form a separate family of carotenoid 1,2-hydratase, and are evolutionarily distant from the known hydroxyneurosporene synthase (CrtC) family.

METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. The wild-type and mutant strains of *D. radiodurans* R1 (ATCC 13939) were grown at 30 °C and the wild-type strain of *D. geothermalis* DSM 11300 was grown at 50 °C in TGY medium [0.5% (w/v) Bacto tryptone, 0.1% (w/v) glucose, and 0.3% (w/v) Bacto yeast extract] on an orbital shaker or on TGY plates solidified with 1.5% (w/v) agar. *Escherichia coli* was cultured in LB medium at 37 °C. Tetracycline (25 µg ml⁻¹), ampicillin (60 µg ml⁻¹), chloramphenicol (30 µg ml⁻¹) or kanamycin (20 µg ml⁻¹) was added to the medium if required.

Sequence and phylogenetic analysis. The gene neighbourhoods of *crtO* homologues in the genomes of *D. radiodurans* R1 and *D. geothermalis* DSM 11300 (<http://ncbi.nlm.nih.gov>) were inspected for gene candidates encoding carotenoid biosynthetic enzymes. Two

nearby sequences (ORFs), *dr0091* and *dgeo2309*, were selected for functional analysis. Their sequences were used to retrieve the homologues in other organisms by the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The known CrtC protein sequences were obtained from the Swiss-Prot database (<http://www.expasy.org/>). Multiple sequences were aligned using the CLUSTAL W program (<http://www.ebi.ac.uk/clustalw/>). The evolutionary tree of protein sequences was constructed by the neighbour-joining (NJ) method with protein p-distances by the MEGA3.1 (Molecular Evolutionary Genetics Analysis) software (<http://www.megasoftware.net/index.html>), and the reliability of the tree topology was assessed by 1000 bootstrap replications.

Construction of expression plasmids. The *dr0091* or *dgeo2309* gene was amplified from *D. radiodurans* and *D. geothermalis* by the primers RKdr91 P1 and RKdr91 P2 or the primers RKdg2309 P1 and RKdg2309 P2, respectively (primer sequences are listed in Supplementary Table S1, available with the online version of this paper). The PCR products were ligated into TA cloning vector pMD18-T. The *Hind*III/*Bam*HI-digested fragments were cloned in the *Hind*III/*Bam*HI site of pRK404, yielding pRK-DR91 or pRK-DG2309 (Table 1).

E. coli transformants containing pRK-DR91 or pRK-DG2309 as well as pACCRT-EBI_{RC} (Albrecht *et al.*, 1997), pACCRT-EBI_{Eu} (Misawa *et al.*, 1995) or pAC-GAMMA (Cunningham & Gantt, 2005) were incubated at 28 °C. IPTG was added to a final concentration of 0.5 mM when the OD₆₀₀ of the culture reached about 0.5.

Construction of mutant strains. Mutants were constructed by double-crossover recombination of a kanamycin-resistance cassette into the genome as previously described (Tian *et al.*, 2007; Xu *et al.*, 2007). The strategy for disrupting *dr0091* is shown in Supplementary Fig. S2(a). The 636 bp upstream and 537 bp downstream fragments of the *dr0091* gene were amplified by the primers M91 P1/M91 P2

Fig. 1. Proposed main steps in the deinoxanthin biosynthetic pathway of *D. radiodurans*. Bold arrows refer to enzymes that have been experimentally confirmed in *D. radiodurans* R1 (CrtB, CrtI, CrtLm, CrtO, CrtD, CruF). Dotted arrows (CrtG-like, GGPP synthase) indicate hypothetical steps catalysed by enzymes that have not yet been identified in this bacterium. FPP, farnesyl diphosphate.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics*	Source or reference
Strains		
<i>Deinococcus radiodurans</i>		
R1	Wild-type (ATCC 13939)	Laboratory stock
R1ΔcruF	<i>D. radiodurans</i> dr0091 gene knockout mutant	This work
<i>Deinococcus geothermalis</i>	Wild-type (DSM 11300)	Makarova <i>et al.</i> (2007)
<i>Escherichia coli</i>		
DH5α	Host for cloning vectors	Laboratory stock
Plasmids		
pMD18-T	TA cloning vector, Ap ^r	Takara
pACCRT-EBI _{Eu}	Produces primarily lycopene in <i>E. coli</i> , Cm ^r	Albrecht <i>et al.</i> (1997)
pACCRT-EBI _{Rc}	Produces primarily neurosporene in <i>E. coli</i> , Cm ^r	Misawa <i>et al.</i> (1995)
pAC-GAMMA	Produces primarily γ-carotene in <i>E. coli</i> , Cm ^r	Cunningham & Gantt (2005)
pRK404	Cloning vector, Tet ^r	Laboratory stock
pRK-DR91	pRK404 containing <i>Hind</i> III– <i>Bam</i> HI fragment of <i>dr0091</i>	This work
pRK-DG2309	pRK404 containing <i>Hind</i> III– <i>Bam</i> HI fragment of <i>dgeo2309</i>	This work
pRADZK	<i>E. coli</i> – <i>D. radiodurans</i> shuttle vector carrying R1 <i>groEL</i> promoter, Ap ^r	Laboratory stock

*Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance.

(containing a *Hind*III restriction site) and M91 P3 (containing a *Bam*HI restriction site)/M91 P4, respectively (Supplementary Table S1). The two fragments, digested by *Hind*III and *Bam*HI, respectively, were ligated to the *Hind*III/*Bam*HI-digested kanamycin gene. The joint product was transformed into *D. radiodurans* R1 by the CaCl₂ method. The mutant strain, in which the DR0091 gene was replaced with a kanamycin-resistance gene, was plated and screened out on TGY agar containing kanamycin, and designated R1ΔcruF. Gene mutation was confirmed by PCR product size analysis (Supplementary Fig. S2b). Furthermore, DNA sequencing of the PCR fragment from the mutant confirmed that the DR0091 gene had been replaced with a kanamycin-resistance cassette in the mutant.

Isolation and analysis of carotenoids. The method used to isolate the carotenoids was as described previously (Xu *et al.*, 2007). A 100 ml sample of each culture (OD₆₀₀ 1.0) grown under aerobic conditions with continuous shaking was harvested by centrifugation at 5000 g for 10 min. After washing three times with sterile water, the cell pellets were extracted three times with cold acetone/methanol (7:2, v/v) in the dark. The pooled carotenoid extracts were directly analysed by HPLC using a Waters 2690 Alliance system as previously described (Tian *et al.*, 2008). A Hypersil ODS-C18 column (4.6 × 250 mm, 5 μm) was used and eluted with acetonitrile/methanol/2-propanol (40:50:10, by vol.) at a flow rate of 1 ml min⁻¹. The separated carotenoids were detected with a Waters 996 photodiode array detector and their absorption spectra were recorded online. The carotenoids were identified by their retention times and absorption spectral features, and by comparison with references (Saito *et al.*, 1998; Steiger *et al.*, 2003; Takaichi & Shimada, 1992). LC-mass spectra of the carotenoids were recorded on an Agilent 1100 series LC/MSD Trap SL mass spectrometer system using atmospheric pressure chemical ionization (APCI). The system was controlled and data were analysed on a computer equipped with LC/MSD Trap Software 4.2 (Bruker). Detection was carried out in the negative ion mode with a corona current of 4.0 μA, a capillary voltage of 2.6 kV, a capillary exit voltage of -129.6 V, a dry temperature of 350 °C, a vaporizer temperature of 425 °C, high-purity (99.999%) dry nitrogen gas at 8.0 l min⁻¹, and a nitrogen nebulizer pressure of 414 kPa.

RESULTS

Identification of candidate genes

Although a gene homologue of known CrtCs was not found in the *D. radiodurans* genome, it seemed likely that a carotenoid 1,2-hydratase of different sequence character would be present in the genome. A CrtC homologue was also not found in the genome sequence of *D. geothermalis* (Makarova *et al.*, 2007). By comparative genomic analysis, the homologues of CrtB, CrtI, CrtLm, CrtO and CrtD in *D. geothermalis* were predicted to be Dego0523, Dgeo0524, Dgeo0857, Dgeo2310 and Dgeo2306, which have 65 %, 79 %, 61 %, 84 % and 73 % identity to those of *D. radiodurans*, respectively. Since carotenoid synthesis genes in some bacteria are assembled in clusters or in neighbourhoods to carry out their functions (Giraud *et al.*, 2004; To *et al.*, 1994), the gene neighbourhood of *crtO* in the genome *D. radiodurans* R1 and *D. geothermalis* DSM 11300 was inspected for gene candidates encoding carotenoid biosynthetic enzymes (Supplementary Fig. S1). We identified two ORFs of unknown function (*dr0091* and *dgeo2309*) in the neighbourhoods of *crtO* (*dr0093*) of *D. radiodurans* and the *crtO* homologue (*dgeo2310*) of *D. geothermalis*, respectively. Since the ORF *dr0092* was predicted to encode a MuT family protein of *D. radiodurans*, it was not selected as the possible *crt* gene candidate. *dr0091* and *dgeo2309* were selected for functional analysis.

Expression of *dr0091* and *dgeo2309* genes in *E. coli* producing neurosporene or lycopene

To characterize *dr0091* and *dgeo2309*, the expression vector pRK-DR91 or pRK-DG2309 was co-transformed with

Fig. 2. Expression of *dr0091* or *dgeo2309* in neurosporene-producing or lycopene-producing strains of *E. coli*. (a) HPLC analysis of carotenoids in neurosporene-producing *E. coli* transformants containing the plasmids pACCRTEBI_{Rc}, pACCRTEBI_{Rc} + pRK-DR91 or pACCRTEBI_{Rc} + pRK-DG2309. (b) HPLC analysis of carotenoids in lycopene-producing *E. coli* transformants containing the plasmids pACCRTEBI_{Eu}, pACCRTEBI_{Eu} + pRK-DR91 or pACCRTEBI_{Eu} + pRK-DG2309. Carotenoids were analysed by reverse-phase HPLC: column, Hypersil C18 (250×4.6 mm, 5 µm, Alltech); elution, acetonitrile/methanol/2-propanol (4:5:1, by vol.); flow rate, 1 ml min⁻¹; detection at 470 nm. Peak 1, 1,1'-(OH)₂-neurosporene; peak 2, 1-OH-neurosporene; peak 3, neurosporene; peak 4, 1,1'-(OH)₂-lycopene; peak 5, 1-OH-lycopene; peak 6, lycopene.

plasmid pACCRTEBI_{Rc} or pACCRTEBI_{Eu}, respectively, into *E. coli* DH5α, which does not produce carotenoids. Carotenoid compositions in *E. coli* transformants were analysed by HPLC and LC-MS (Fig. 2, Table 2). *E. coli* containing pACCRTEBI_{Rc} produced only neurosporene (peak 3 in Fig. 2a). When the *dr0091* and *dgeo2309* genes were expressed in the neurosporene-producing strain of *E. coli*, two other carotenoid products were observed (peaks 1 and 2 in Fig. 2a). The shorter retention times of peak 1 and peak 2 indicated that these products were more hydrophilic

than neurosporene. Based on the retention time, absorbance spectrum and molecular mass of the products (Table 2), peak 1 (λ_{max} 414, 439, 468 nm in HPLC mobile phase) with a mass of 574 Da and peak 2 (λ_{max} 414, 439, 468 nm) with a mass of 556 Da were identified as 1,1'-(OH)₂-neurosporene and 1-OH-neurosporene, respectively (Steiger *et al.*, 2003). *E. coli* containing pACCRTEBI_{Eu} produced only lycopene (peak 6 in Fig. 2b). Expression of the *dr0091* and *dgeo2309* genes in the lycopene-producing strain of *E. coli* resulted in the appearance of peak 4 (λ_{max} 445, 470, 502 nm) with a mass of 572 Da and peak 5 (λ_{max} 445, 471, 502 nm) with a mass of 554 Da (Fig. 2b; Table 2). Peaks 4 and 5 were identified as 1,1'-(OH)₂-lycopene and 1-OH-lycopene (Steiger *et al.*, 2003). These results demonstrated that *dr0091* and *dgeo2309* encode a carotenoid 1,2-hydrotase that can not only convert neurosporene or lycopene into 1-OH-neurosporene or 1-OH-lycopene, but also convert the monohydroxylated carotenoids 1-OH-neurosporene or 1-OH-lycopene into 1,1'-(OH)₂-neurosporene or 1,1'-(OH)₂-lycopene.

Expression of *dr0091* and *dgeo2309* genes in *E. coli* producing γ -carotene

To further analyse the substrate specificity of the *dr0091* and *dgeo2309* gene products, the expression vector pRK-DR91 or pRK-DG2309 was co-transformed with the plasmid pAC-GAMMA into *E. coli* DH5α. *E. coli* containing pAC-GAMMA produced γ -carotene (peak 8 in Fig. 3) and β -carotene (peak 9 in Fig. 3). Expression of *dr0091* and *dgeo2309* in *E. coli* containing pAC-GAMMA resulted in the generation of a more hydrophilic product, peak 7 (8 min; λ_{max} 461, 490 nm; 554 Da) (Fig. 3 and Table 2). Based on the retention time, absorbance spectrum and molecular mass, peak 7 was identified as 1'-OH- γ -carotene (Frigaard *et al.*, 2004). These results demonstrated that DR0091 and Dgeo2309 can also convert monocyclic γ -carotene into 1'-OH- γ -carotene.

Mutant construction and carotenoid composition analysis of the mutant

The strategy for disrupting *dr0091* is shown in Supplementary Fig. S2(a). Mutation of *dr0091* resulted in a pink strain, designated R1ΔcruF. The carotenoid composition of the mutant was analysed by HPLC and compared with the wild-type R1. As shown in Fig. 4(a), peak 1' in mutant R1ΔcruF had a retention time of 5.3 min and a λ_{max} of 468 nm and 490 nm in the mobile phase (Fig. 4c), while deinoxanthin (peak 1 in wild-type R1) had a shorter retention time, and a λ_{max} of 479 nm and 507 nm (Fig. 4b). The longer retention time and the spectral absorption blue shift of peak 1' relative to deinoxanthin indicated that peak 1' was less hydrophilic and had fewer conjugated bonds (Takaichi & Shimada, 1992; Takaichi, 2000). The absorbance spectrum of peak 1' was similar to 3',4'-dihydrodeinoxanthin (C₄₀H₅₆O₃), the major product of the mutant of carotenoid 3',4'-desaturase (CrtD) (Tian

Table 2. Carotenoid composition in *E. coli* transformants carrying various plasmids

Plasmid	Carotenoids accumulated*	Retention time (min)	Mol. mass (Da)	Spectroscopic properties [$\lambda_{\max}$ (nm) in HPLC eluent]†
pACCRT-EBI _{Rc}	Neurosporene	13.2	538	416, 440, 468
pACCRT-EBI _{Rc} + pRK-DR91	1,1'-(OH) ₂ -neurosporene	4.4	574	414, 439, 468
	1-OH-neurosporene	7.3	556	414, 439, 468
	Neurosporene			
pACCRT-EBI _{Rc} + pRK-DG2309	1,1'-(OH) ₂ -neurosporene	4.4	574	414, 439, 468
	1-OH-neurosporene	7.3	556	414, 439, 468
	Neurosporene			
pACCRT-EBI _{Eu}	Lycopene	11.5	536	445, 471, 502
pACCRT-EBI _{Eu} + pRK-DR91	1,1'-(OH) ₂ -lycopene	4.1	572	445, 470, 502
	1-OH-lycopene	6.6	554	445, 471, 502
	Lycopene			
pACCRT-EBI _{Eu} + pRK-DG2309	1,1'-(OH) ₂ -lycopene	4.1	572	445, 470, 502
	1-OH-lycopene	6.6	554	445, 471, 502
	Lycopene			
pAC-GAMMA	γ -Carotene	14.2	536	(435), 461, 491
	β -Carotene	17.5	536	(424), 452, 479
pAC-GAMMA + pRK-DR91	1'-OH- γ -carotene	8.0	554	(435), 461, 490
	γ -Carotene, β -carotene			
pAC-GAMMA + pRK-DG2309	1'-OH- γ -carotene	8.0	554	(435), 461, 490
	γ -Carotene, β -carotene			

*Carotenoids were analysed by reverse-phase HPLC: column, 5 μ m Hypersil C18 (250 $\times$ 4.6 mm, Alltech); elution, acetonitrile/methanol/2-propanol (4:5:1, by vol.); flow rate, 1 ml min⁻¹; detection, 470 nm.

†Wavelengths in parentheses indicate a shoulder rather than a distinct peak.

et al., 2008). From its main absorption maximum ($\lambda_{\max}$ 468 nm in HPLC eluate), peak 1' was suggestive of 12 conjugated double bonds including a conjugated keto group (Takaichi & Shimada, 1992; Takaichi, 2000). Mass analysis showed peak 1' with a mass of 566 Da (matching

Fig. 3. Expression of the *dr0091* or *dgeo2309* in a γ -carotene-producing strain of *E. coli*: HPLC analysis of carotenoids in *E. coli* transformants containing the plasmids pAC-GAMMA, pAC-GAMMA + pRK-DR91 or pAC-GAMMA + pRK-DG2309. Peak 7, 1'-OH- γ -carotene; peak 8, γ -carotene; peak 9, β -carotene.

the formula C₄₀H₅₄O₂). The spectral properties and mass result suggested that peak 1' was 2-hydroxy-4-keto- γ -carotene, which is related to 3',4'-dihydrodeinoxanthin but lacks a hydration modification. Our previous study showed that CrtD (DR2250) in *D. radiodurans* cannot catalyse C-3',4' desaturation of γ -carotene derivatives without the hydration reaction at the C-1',2' double bond (Tian *et al.*, 2008). Therefore, DR0091 was confirmed to be a carotenoid 1,2-hydratase, which catalyses the hydration reaction before the C-3',4' desaturation step by CrtD (Fig. 1).

Peak 2' in mutant R1 Δ cruF had a retention time of 7.25 min with a $\lambda_{\max}$ of 468 nm and 486 nm in the mobile phase (Fig. 4d). Peak 2' showed a mass of 550 Da (matching the formula C₄₀H₅₄O). Based on the absorbance spectrum, mass and reference (Sandmann *et al.*, 2006), peak 2' was identified as 4-keto- γ -carotene. Peak 3' showed the same retention time (14.2 min) and absorbance spectrum ($\lambda_{\max}$ 461, 491 nm) (Fig. 4e) as the reference γ -carotene (Table 2; peak 8 in Fig. 3); therefore, peak 3' was identified as γ -carotene. Lycopene or earlier intermediates were not detected in the mutant strain, suggesting that DR0091 cannot catalyse these acyclic substrates in the native host cell.

Phylogenetic analysis of carotenoid 1,2-hydratase

Sequences of DR0091 homologues were retrieved using the BLAST program (<http://www.ncbi.nlm.nih.gov/>). DR0091

Fig. 4. Analysis of carotenoids from wild-type R1 and mutant R1ΔcruF. (a) HPLC elution profile of carotenoids from wild-type R1 and mutant R1ΔcruF. (b–e) Absorption spectra of identified peaks. Peak 1, deinoxanthin (b); peak 1', 2-hydroxy-4-keto-γ-carotene (c); peak 2', 4-keto-γ-carotene (d); peak 3', γ-carotene (e).

showed 63% identity to Dgeo2309. Their homologues found in carotenoid-producing bacteria were present in the DUF422 superfamily. The DR0091 amino acid sequence was found to be 35% identical to that of carotenoid hydratase (CruF) from *Synechococcus* sp. PCC 7002 (Maresca *et al.*, 2008). Phylogenetic analysis showed that DR0091, Dgeo2309 and other CruF homologues were closer to each other than to CrtC homologues (Fig. 5). DR0091 and Dgeo2309 together with other CruF homologues formed a separate family distant from the known CrtC family.

DISCUSSION

Carotenoid 1,2-hydratase, a carotenoid biosynthetic enzyme, is required to catalyse the synthesis of deinoxanthin by hydration at C-1',2'. In the present study, we identified two ORFs of previously unknown function (*dr0091* and *dgeo2309*) as gene candidates of carotenoid

1,2-hydratase in the neighbourhoods of *crtO* of *D. radiodurans* and the *crtO* homologue of *D. geothermalis*, respectively. Combining gene expression and disruption analysis, we confirmed that *dr0091* and *dgeo2309* encode a novel carotenoid 1,2-hydratase in *D. radiodurans* and *D. geothermalis*. The genes *dr0091* and *dgeo2309* showed little homology to the CrtC-type carotenoid 1,2-hydratase, which is found mainly in photosynthetic bacteria (Armstrong *et al.*, 1989; Frigaard *et al.*, 2004; Giraud *et al.*, 2004; Kovács *et al.*, 2003; Lang *et al.*, 1995; Ouchane *et al.*, 1997; Scolnik *et al.*, 1980) and in the non-photosynthetic bacterium *Myxococcus xanthus* (Botella *et al.*, 1995). Recently, a carotenoid 1,2-hydratase homologue of DR0091 and Dgeo2309 from the cyanobacterium *Synechococcus* sp. PCC 7002 was described and named CruF in a review (Maresca *et al.*, 2008). This enzyme was shown to be required for the first committed step in myxoxanthophyll synthesis, and the hydration catalysed by the CruF from *Synechococcus* sp. PCC 7002 could be introduced both on acyclic lycopene and on monocyclic

Fig. 5. Neighbour-joining phylogenetic tree of carotenoid 1,2-hydratases. Sequences analysed: *Deinococcus radiodurans* R1 DR0091 (NP_293817.1), *Deinococcus geothermalis* DSM 11300 Dgeo2309 (YP_605770.1), *Synechococcus* sp. PCC 7002 CruF (YP_001735274.1), *Gloeobacter violaceus* PCC 7421 Glr1356 (NP_924302.1), *Thermosynechococcus elongatus* BP-1 Tlr0414 (NP_681204.1), *Anabaena variabilis* ATCC 29413 Ava_1513 (YP_322031.1), *Synechocystis* sp. PCC 6803 Sll0814 (NP_441438.1), *Nostoc punctiforme* PCC 73102 Npun_R6006 (YP_001869239.1), *Salinibacter ruber* DSM 13855 SRU1431 (YP_445555.1), *Frankia alni* ACN14a FRAAL2170 (YP_712398.1), *Myxococcus xanthus* DK1050 CrtC (CAA79959.1), *Pelodictyon luteolum* DSM 273 CrtC (Q3B257), *Chlorobium ferrooxidans* DSM 13031 CrtC (Q0YRY6), *Chlorobaculum tepidum* TLS CrtC (NP_661205.1), *Roseobacter denitrificans* OCh114 CrtC (Q16DU2), *Rhodobacter sphaeroides* 2.4.1 CrtC (YP_353342.1), *Rhodobacter capsulatus* CrtC (P17058), *Rhodospirillum rubrum* S1 CrtC (AAN75036.1), *Thiocapsa roseopersicina* BBS CrtC (Q7X3G5), *Rubrivivax gelatinosus* S1 CrtC (P95619), *Bradyrhizobium* sp. ORS278 CrtC (YP_001203729.1) and *Rhodopseudomonas palustris* CGA009 CrtC (NP_946865.1).

γ -carotene (Graham & Bryant, 2009). The authors reported that homologues of CruF were also detected in the genomes of all sequenced cyanobacterial strains that produce myxol or the acyclic xanthophyll oscillaxanthin. Cyclization of lycopene by lycopene cyclase may be either before or after the hydroxylation step catalysed by the CruF from *Synechococcus* sp. PCC 7002. However, in our analysis of the carotenoid intermediates in the mutant R1 Δ cruF, no acyclic lycopene derivatives or earlier intermediates were detected in the mutant, suggesting that DR0091 cannot catalyse the acyclic substrates in the native host. The CruF in *D. radiodurans* may catalyse the hydration reaction at the C-1',2' double bond of the monocyclic precursor of deinoxanthin. This result is consistent with our previous work on a mutant of lycopene cyclase (DR0801), in which no hydroxylated products of lycopene were detected, only lycopene, confirming that substituents on the end group including C-1',2' hydration and C-3',4' desaturation should be added after the cyclization of lycopene (Tian *et al.*, 2008). To our knowledge, this is the first report of a functional carotenoid 1,2-hydratase (CruF) in non-photosynthetic bacteria. The identification of the CruF from *D. geothermalis* indicated that the major carotenoids might be

hydroxylcarotenoids with C1 (or 1') hydroxyl groups. However, two major carotenoid products with retention times (13.5 min and 15.4 min) different from deinoxanthin (3.4 min) were found in *D. geothermalis* using the same HPLC conditions (unpublished data). This indicated that the carotenoid composition and biosynthetic pathway of *D. geothermalis* may be different from that of *D. radiodurans*.

The functional analysis of DR0091 in the present study helped to reveal the deinoxanthin biosynthetic pathway in *D. radiodurans*. Disruption of the *dr0091* gene (*cruF*) not only blocked the hydration at C-1',2', but also inactivated the desaturation at C-3',4'. This result was consistent with our previous findings that the C-3',4' desaturation was catalysed by CrtD (DR2250) following a hydration reaction at C-1',2' (Tian *et al.*, 2008). Another carotenogenic enzyme in the proposed pathway not yet identified in *D. radiodurans* is 2-hydroxylase. A carotenoid 2,2'- β -hydroxylase (CrtG) was detected in *Brevundimonas* sp. strain SD212 (Nishida *et al.*, 2005). Recently, a CrtG was found in the carotenoid biosynthetic pathway of *Thermosynechococcus elongatus* strain BP-1; this was the first functional identification of CrtG in a cyanobacterium (Iwai *et al.*,

2008). However, no homologues of this gene have been found in the genome of *D. radiodurans*.

The phylogenetic tree (Fig. 5) demonstrated the close evolutionary relationship among the CruF homologues. A similar close evolutionary relationship was also shown between the lycopene cyclase (CrtLm) from *D. radiodurans* and the CrtL from *Synechococcus* sp. PCC 7942 (Krubasik & Sandmann, 2000). The CruF homologues formed a separate clade of carotenoid 1,2-hydratase, and are evolutionarily distant from the known hydroxyneurosporene synthase (CrtC) clade. Since cyanobacteria and non-photosynthetic *Deinococcus* are grouped in the same cluster, it may be inferred that lateral gene transfer has occurred between these bacteria. It has been suggested that lateral gene transfer events may have made a substantial contribution to the distribution of genes for carotenoid biosynthesis across bacterial genomes (Phadwal, 2005).

The antioxidant capacity of carotenoids is thought to be linked to the length of their conjugated double bond system and the presence of functional groups (Albrecht *et al.*, 2000). Besides an extension of the conjugated double bond, there is an additional hydroxyl group substitution at C-1' of the double bonds in deinoxanthin. The presence of the hydroxyl group may enhance its scavenging activity by hydrogen abstraction reactions with ROS. Hydroxyllycopene has been reported to have greater antioxidant properties than lycopene because of the presence of a hydroxyl group at position C-1' (Albrecht *et al.*, 2000). Therefore, carotenoid 1,2-hydratase (DR0091), responsible for the hydration at C-1',2' in deinoxanthin biosynthesis, is important for the antioxidant activity of deinoxanthin. As non-enzymic ROS scavengers, carotenoids in *D. radiodurans* may act together with pyrroloquinoline quinone (Misra *et al.*, 2004) and a high intracellular Mn(II) to Fe(II) ratio (Daly *et al.*, 2007) in intracellular resistance against oxidative stress.

In this study, we identified and characterized a novel carotenoid 1,2-hydratase (CruF) in the non-photosynthetic bacteria *D. radiodurans* and *D. geothermalis*. We also determined the possible reaction sequences involved in deinoxanthin biosynthesis. The carotenoid biosynthetic enzymes identified from *Deinococcus* can also be used, via genetic engineering, for the production of novel carotenoids with high activities. Further investigations of the remaining unidentified carotenoid biosynthetic enzymes are needed to elucidate the carotenoid biosynthetic pathway in *Deinococcus* fully.

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