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

Deinococcus

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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 (carotenoids) 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 disphosphate (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 (CrtB). Phytoene is then converted to lycopene by phytoene desaturase (CrtI, DR0861) and lycopene cyclase (CrtLm), deinoxanthin by hydration at the C-1 end group (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.
from \textit{D. radiodurans} R1 and \textit{D. geothermalis} DSM 11300, respectively. The homologue of \textit{dr}0091 and \textit{dgeo}2309 from the cyanobacterium \textit{Synechococcus} sp. PCC 7002 was recently described and named CruF by Maresca \textit{et al.} (2008).

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

\section*{METHODS}

\subsection*{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 \textit{D. radiodurans} R1 (ATCC 13939) were grown at 30 °C and the wild-type strain of \textit{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. \textit{Escherichia coli} was cultured in LB medium at 37 °C. Tetracycline (25 mg ml\textsuperscript{-1}), ampicillin (60 \mu g ml\textsuperscript{-1}), chloramphenicol (30 \mu g ml\textsuperscript{-1}) or kanamycin (20 \mu g ml\textsuperscript{-1}) was added to the medium if required.

\subsection*{Sequence and phylogenetic analysis.} The gene neighbourhoods of \textit{crtO} homologues in the genomes of \textit{D. radiodurans} R1 and \textit{D. geothermalis} DSM 11300 (http://ncbi.nlm.nih.gov) were inspected for gene candidates encoding carotenoid biosynthetic enzymes. Two nearby sequences (ORFs), \textit{dr}0091 and \textit{dgeo}2309, were selected for functional analysis. Their sequences were used to retrieve the homologues in other organisms by the \textsc{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 \textsc{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 \textsc{mega}3 (Molecular Evolutionary Genetics Analysis) software (http://www.megasoftware.net/index.html), and the reliability of the tree topology was assessed by 1000 bootstrap replications.

\subsection*{Construction of expression plasmids.} The \textit{dr}0091 or \textit{dgeo}2309 gene was amplified from \textit{D. radiodurans} and \textit{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 \textsc{Hind}{\textsc{III}}/\textsc{Bam}{\textsc{HI}}-digested fragments were cloned in the \textsc{Hind}{\textsc{III}}/\textsc{Bam}{\textsc{HI}} site of pRK404, yielding pRK-DR91 or pRK-DG2309 (Table 1).

\subsection*{Construction of mutant strains.} Mutants were constructed by double-crossover recombination of a kanamycin-resistance cassette into the genome as previously described (Tian \textit{et al.}, 2007; Xu \textit{et al.}, 2007). The strategy for disrupting \textit{dr}0091 is shown in Supplementary Fig. S2(a). The 636 bp upstream and 537 bp downstream fragments of the \textit{dr}0091 gene were amplified by the primers M91 P1/M91 P2.
et al. were directly analysed by HPLC using a Waters 2690 Alliance system with a Waters 2996 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 published data (Saito, 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 1 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 cto 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 cto. Although a gene homologue of known CrtCs was not found in the genome of D. radiodurans, it was not selected as the possible cto 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
plasmid pACCRT-EBI_{Rc} or pACCRT-EBI_{Eu} respectively, into E. coli DH5\(\alpha\), 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 pACCRT-EBI_{Rc} produced only neurosporene, based on the retention time, absorbance spectrum and molecular mass of the products (Table 2), peak 1 (\(\lambda_{\text{max}}\) 414, 439, 468 nm in HPLC mobile phase) with a mass of 574 Da and peak 2 (\(\lambda_{\text{max}}\) 414, 439, 468 nm) with a mass of 556 Da were identified as 1,1',-(OH)\(_2\)-neurosporene and 1-\(\text{OH}\)-neurosporene, respectively (Steiger et al., 2003). E. coli containing pACCRT-EBI_{Eu} produced only lycopene (peak 6 in Fig. 2b). Expression of the\textit{dr0091} and\textit{dgeo2309} genes in the lycopene-producing strain of E. coli resulted in the appearance of peak 4 (\(\lambda_{\text{max}}\) 445, 470, 502 nm) with a mass of 572 Da and peak 5 (\(\lambda_{\text{max}}\) 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-\(\text{OH}\)-lycopene (Steiger et al., 2003), respectively. These results demonstrated that\textit{dr0091} and \textit{dgeo2309} encode a carotenoid 1,2-hydrazase that can not only convert neurosporene or lycopene into 1-\(\text{OH}\)-neurosporene or 1-\(\text{OH}\)-lycopene, but also convert the monohydroxylated carotenoids 1-\(\text{OH}\)-neurosporene or 1-\(\text{OH}\)-lycopene into 1,1',-(OH)\(_2\)-neurosporene or 1,1',-(OH)\(_2\)-lycopene.

Expression of\textit{dr0091} and\textit{dgeo2309} genes in\textit{E. coli} producing \(\gamma\)-carotene

To further analyse the substrate specificity of the \textit{dr0091} and \textit{dgeo2309} gene products, the expression vector pRK\textit{-DG2309} was co-transformed with the plasmid pAC-GAMMA into E. coli DH5\(\alpha\). E. coli containing pAC-GAMMA produced \(\gamma\)-carotene (peak 8 in Fig. 3) and \(\beta\)-carotene (peak 9 in Fig. 3). Expression of\textit{dr0091} and \textit{dgeo2309} in E. coli containing pAC-GAMMA resulted in the generation of a more hydrophilic product, peak 7 (8 min; \(\lambda_{\text{max}}\) 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-\(\text{OH}\)-\(\gamma\)-carotene (Frigaard et al., 2004). These results demonstrated that DR0091 and Dgeo2309 can also convert monocyclic \(\gamma\)-carotene into 1-\(\text{OH}\)-\(\gamma\)-carotene.

Mutant construction and carotenoid composition analysis of the mutant

The strategy for disrupting \textit{dr0091} is shown in Supplementary Fig. S2(a). Mutation of \textit{dr0091} resulted in a pink strain, designated R1\textit{AcruF}. 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 R1\textit{AcruF} had a retention time of 5.3 min and a \(\lambda_{\text{max}}\) 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 \(\lambda_{\text{max}}\) 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 (C\(_{40}\)H\(_{62}\)O\(_3\)), the major product of the mutant of carotenoid 3’,4’-\textit{desaturase} (CrtD) (Tian
From its main absorption maximum ($\lambda_{\text{max}}$ 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 the formula C$_{40}$H$_{54}$O$_2$). The spectral properties and mass result suggested that peak 1 was 2-hydroxy-4-keto-$\gamma$-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 $\gamma$-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 R1AcruF had a retention time of 7.25 min with a $\lambda_{\text{max}}$ of 468 nm and 486 nm in the mobile phase (Fig. 4d). Peak 2 showed a mass of 550 Da (matching the formula C$_{40}$H$_{54}$O). Based on the absorbance spectrum, mass and reference (Sandmann et al., 2006), peak 2 was identified as 4-keto-$\gamma$-carotene. Peak 3 showed the same retention time (14.2 min) and absorbance spectrum ($\lambda_{\text{max}}$ 461, 491 nm) (Fig. 4e) as the reference $\gamma$-carotene (Table 2; peak 8 in Fig. 3); therefore, peak 3 was identified as $\gamma$-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.

**Fig. 3.** Expression of the dr0091 or dgeo2309 in a $\gamma$-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-$\gamma$-carotene; peak 8, $\gamma$-carotene; peak 9, $\beta$-carotene.

### 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. 3](http://mic.sgmjournals.org)
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; Kovačs 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...
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 R1DcruF, 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 Cl (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 dru091 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'-b-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., 2009).
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 CrucF 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 CrucF homologues formed a separate clade of carotenoid 1,2-hydratase, and are evolutionarily distant from the known hydroxyneuropsen 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. Hydroxylcopene 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. goethalmis*. 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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