Carotenoid 3',4'-desaturase is involved in carotenoid biosynthesis in the radioresistant bacterium *Deinococcus radiodurans*

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Deinococcus radiodurans strain R1 synthesizes deinoxanthin, a unique carotenoid product, which contributes to cell resistance following various stresses. The biosynthetic pathway of deinoxanthin is unclear, although several enzymes are presumed to be involved. The gene (*dr2250*) predicted by gene homologue analysis to encode carotenoid 3',4'-desaturase (CrtD) was deleted to investigate its function. A mutant deficient in the gene homologue of *crtLm* (*dr0801*) was also constructed to verify the catalytic function of the gene product in the native host. Carotenoid analysis of the resultant mutants verified that DR2250 encodes carotenoid 3',4'-desaturase, which catalyses the C-3',4'-desaturation of the monocyclic precursor of deinoxanthin but not acyclic carotenoids. Mutation of the gene homologue of *crtLm* (*dr0801*) resulted in accumulation of lycopene, confirming that it encodes the lycopene cyclase in the native host. The lack of CrtD decreased the antioxidant capacity of the mutant deficient in *dr2250* compared with the wild-type, indicating that the C-3',4'-desaturation step contributes to the antioxidant capacity of deinoxanthin in *D. radiodurans*.

Received 4 June 2008

Revised 8 September 2008

Accepted 9 September 2008

INTRODUCTION

Carotenoids are known to scavenge reactive oxygen species (ROS), and are synthesized in various organisms including bacteria and plants (Armstrong & Hearst, 1996; Fraser & Bramley, 2004; Misawa *et al.*, 1990; Krinsky & Johnson, 2005). Generally, carotenoid biosynthesis starts with the synthesis of phytoene by the condensation of two molecules of geranylgeranyl diphosphate. This step is followed by desaturation catalysed by bacterial-type phytoene desaturase (CrtI), resulting in the synthesis of lycopene (Armstrong *et al.*, 1990; Harada *et al.*, 2001), or by cyanobacterial-type phytoene desaturase (CrtP), resulting in the production of ζ -carotene (Martínez-Férez & Vioque, 1992). Further modification reactions, including cyclization, ketolation or hydroxylation, lead to the formation of different carotenoid products.

Deinococcus radiodurans is a red-pigmented, non-photosynthetic bacterium well known for its resistance to radiation, oxidants and desiccation (Cox & Battista, 2005).

Abbreviations: APCI, atmospheric pressure chemical ionization; DCFH-DA, 2',7'-dichlorohydrofluorescein diacetate; PQQ, pyrroloquinoline quinone; ROS, reactive oxygen species.

Details of plasmid construction and absorbance spectra of peaks 2 and 2' are available as supplementary material with the online version of this paper.

Antioxidants play an important role in the resistance of *D. radiodurans* (Markillie *et al.*, 1999). The role of the antioxidant metabolite pyrroloquinoline quinone (PQQ) from *D. radiodurans* has been reported (Khairnar *et al.*, 2003). Recently, Daly *et al.* (2004) found that Mn(II) accumulation (with low Fe) might contribute to the resistance of *D. radiodurans* to oxidative stress. *D. radiodurans* strain R1 synthesizes a unique carotenoid which was identified as deinoxanthin (Lemee *et al.*, 1997; Saito *et al.*, 1998). The absolute configuration of the chiral C-2 of deinoxanthin was assigned as *R* (Saito *et al.*, 1998). However, the biosynthetic pathway of deinoxanthin is not well characterized. Several carotenoid biosynthetic enzymes should be encoded in the genome of *D. radiodurans* strain R1 (White *et al.*, 1999), inferred from the chemical structure of deinoxanthin and genome homology analysis (Makarova *et al.*, 2001). We have previously identified phytoene synthase (CrtB, DR0862) and phytoene desaturase (CrtI, DR0861), which are involved in the early steps of deinoxanthin biosynthesis (Tian *et al.*, 2007; Xu *et al.*, 2007). Zhang *et al.* (2007) confirmed the functions of these two genes. Two other genes encoding carotenoid ketolase (CrtO) and lycopene cyclase (CrtLm) in *D. radiodurans* were described (Tao & Cheng, 2004; Tao *et al.*, 2004); however, the steps that they catalyse in the native host are unclear. The biosynthesis of deinoxanthin requires a carotenoid desaturase (CrtD) to

introduce one double bond to position C-3',4'. An open reading frame (*dr2250*) was predicted to encode a methoxy-neurosporene desaturase, but no experimental confirmation of such a function is available. The CrtD from purple bacteria catalyses the C-3,4 desaturation of acyclic carotenoids (Bartley *et al.*, 1990; Lang *et al.*, 1995; Steiger *et al.*, 2000; Kovács *et al.*, 2003). It is not clear whether the C-3',4' desaturation in *D. radiodurans* is introduced into acyclic carotenoids or into cyclic carotenoids. Further work is needed on related genes to elucidate the reaction sequence of the deinoxanthin biosynthetic pathway.

By using comparative genomic analysis, gene mutation and carotenoid analysis, we have identified that *dr2250* encodes carotenoid 3',4'-desaturase, which catalyses the desaturation of monocyclic carotenoids. DR0801 was confirmed to be lycopene cyclase in the native host. These results help to reveal the deinoxanthin biosynthesis pathway, in which the main steps are proposed (Fig. 1). We have also investigated the role of C-3',4'-desaturation in establishing the anti-oxidant activity of deinoxanthin.

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 in TGY medium [0.5% (w/v) Bacto tryptone, 0.1% (w/v) glucose, 0.3% (w/v) Bacto yeast extract] at 30 °C on an orbital shaker or on TGY plates solidified with 1.5% (w/v) agar.

Sequence analysis. Gene homologue sequences were retrieved using the BLAST program (<http://www.ncbi.nlm.nih.gov>). The sequence of the CrtD homologue (DR2250) was obtained from the genome sequence of *D. radiodurans* R1 (<http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?database=gdr>) by sequence similarity searching, using the protein sequence of CrtD from *Rhodobacter capsulatus* (Bartley *et al.*, 1990). The sequence of the CrtLm homologue (DR0801) had been identified in the genomic sequence of *D. radiodurans* (Tao *et al.*, 2004). Multiple sequence alignment was performed with the CLUSTAL W program at the EMBL website (<http://www.ebi.ac.uk>). Conserved regions were analysed by the PROSITE method (<http://www.expasy.org>).

Construction of mutant strains. Mutants were constructed by double-crossover recombination of a kanamycin-resistance cassette into the genome as described previously (Tian *et al.*, 2007; Xu *et al.*, 2007) (Supplementary Fig. S1a, available with the online version of this article). For the construction of a mutant deleted in DR2250, a PCR product carrying the *dr2250* deletion cassette was generated from three ligated PCR DNA fragments: a kanamycin-resistance gene fragment containing a fusion of the *D. radiodurans* *groEL* promoter obtained from pRADK (Gao *et al.*, 2005), which was modified from pRADZ3 (Meima & Lidstrom, 2000), a 981 bp DNA fragment immediately upstream of the *dr2250* initiation codon amplified with primers AF1 (5'-TTCTATTCTTAGGCTGCTCAGACTCCCG T-3') and AR1 (5'-TATTATGGATCCTTTTGCCTGCACTAGGC-3') (*Bam*HI site underlined) and a 1090 bp DNA fragment immediately downstream of the *dr2250* termination codon generated with primers AF3 (5'-TGAGGTAAGCTTGCTTTCTCTCAAGGTCCA-3') (*Hind*III site underlined) and AR3 (5'-GTGTGTGTGGTCACTCAACCGC-TCTTATC-3'). The upstream and downstream fragments were digested with *Bam*HI and *Hind*III, respectively, and ligated to the kanamycin-resistance cassette pretreated with *Bam*HI and *Hind*III. The ligated product was used as a template for PCR with primers AF1

Fig. 1. Proposed carotenoid biosynthetic pathway and the functions of individual carotenoid biosynthetic enzymes in *Deinococcus radiodurans*. Solid arrows refer to enzymes that have been experimentally confirmed in *D. radiodurans* R1 (CrtB, CrtI, CrtLm, CrtO, CrtD). Dotted arrows indicate hypothetical steps catalysed by enzymes that have not yet been identified in this bacterium.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
<i>D. radiodurans</i>		
R1	Wild-type (ATCC 13939)	Laboratory stock
R1ΔcrtD	<i>D. radiodurans</i> DR2250 gene-deletion mutant	This work
R1ΔcrtLm	<i>D. radiodurans</i> DR0801 gene-deletion mutant	This work
Plasmid		
pRADZ3	<i>E. coli</i> - <i>D. radiodurans</i> shuttle vector (Ap ^r , Cm ^r)	Meima & Lidstrom (2000)
pRADK	pRADZ3 derivative in which <i>lacZ</i> is replaced by the kanamycin-resistance gene (Ap ^r , Km ^r , Cm ^r)	Gao <i>et al.</i> (2005)

and AR3. The resulting PCR fragment was transformed into *D. radiodurans* R1 using the CaCl₂ technique. The mutant strain, in which the DR2250 gene was replaced with a kanamycin-resistance gene, was plated and screened out on TGY agar containing kanamycin, and designated R1ΔcrtD. Gene mutation was confirmed by PCR product size analysis and DNA sequencing. PCR across the site of gene replacement (mutation) demonstrated that the wild-type and mutant alleles for the mutant had segregated completely (Supplementary Fig. S1b). Furthermore, DNA sequencing of the PCR fragment from the mutant confirmed that the DR2250 gene had been replaced with a kanamycin-resistance cassette.

For the deletion mutant of the lycopene cyclase homologue gene (*dr0801*), the construction method was the same as that for R1ΔcrtD, except for the PCR primers: an 860 bp DNA fragment immediately upstream of the *dr0801* initiation codon was amplified with primers BF1 (5'-TTGTATGATGCTCGCCCTGAAAACCACCAC-3') and BR1 (5'-TTATAAGGATCCCGGCTCCACCCACGGAA-3') (*Bam*HI site underlined) and a 911 bp sequence immediately downstream of the *dr0801* termination codon was generated with primers BF3 (5'-TTGTTGAAGCTTGTGTCTCAGGCTAAAGCG-3') (*Hind*III site underlined) and BR3 (5'-TTGCTATTGTTGAACCCGAAGCCAC-CCAAC-3'). The resultant mutant strain was designated R1ΔcrtLm. The PCR products generated from the wild-type and mutant using primers BF1 and BR3 were digested with *Nde*I, confirming that the wild-type and mutant alleles for the mutant had segregated completely (Supplementary Fig. S1c).

Isolation and analysis of carotenoids. Cells were harvested by centrifugation at 5000 *g* for 10 min from 50 ml samples of cultures (OD₆₀₀ 1.0) of the wild-type and mutants grown under aerobic conditions with continuous shaking. After washing three times with sterile water, the cell pellet was extracted three times with cool acetone/methanol (7:2, v/v) in the dark. The pooled carotenoid extracts were analysed directly by HPLC using a Waters 2690 Alliance system as described previously (Tian *et al.*, 2007). A Hypersil ODS-C18 column (4.6 × 250 mm, 5 μm) was used and eluted with a mixture of acetonitrile, methanol and 2-propanol (40:50:10, v/v) at a flow rate of 0.8 ml min⁻¹. The separated carotenoids were detected with a Waters 996 photodiode array detector and their absorption spectra were recorded online. To investigate the effect of different solvents on absorption spectra, we also measured the absorption spectra of the major carotenoids in acetone, ethanol or methanol, using a Pharmacia Ultrospec-2000 spectrophotometer. Carbonyl groups in carotenoids were detected by reduction treatment with 5% NaBH₄ in ethanol (Saito *et al.*, 1998). The carotenoids were identified by their retention time and absorption spectral features and were compared with references (Takaichi & Shimada, 1992; Lemee *et al.*, 1997; Saito *et al.*, 1998). The total carotenoid content [μg (g dry cell weight)⁻¹] was determined using the mean *E*_{1%^{1cm}} (2500) of the main absorption maximum in acetone (Saito *et al.*, 1998). LC-mass

spectra of 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, a high purity (99.999%) dry nitrogen gas of 8.0 l min⁻¹ and a nitrogen nebulizer pressure of 414 kPa (Jin *et al.*, 2007). Deinoxanthin from the wild-type strain and the major carotenoid from R1ΔcrtD were purified using TLC and column chromatography as described by Lemee *et al.* (1997). Lycopene and astaxanthin were obtained from Sigma.

Bacterial survival curves under oxidative stress. Bacterial cells grown to early stationary phase (OD₆₀₀ 1.0) were harvested and suspended in sterile PBS. H₂O₂ (30%) was added to the cell cultures to increasingly greater final concentrations. The cells were treated with H₂O₂ for 90 min. After incubation, the cells were harvested by centrifugation and washed three times with 10 mM phosphate buffer. The cell pellets were diluted and plated on TGY agar plates (Tian *et al.*, 2007). All the plates were incubated at 30 °C for 3 days before surviving colonies were scored. All tests were repeated four times. Survival rates were expressed as the percentage of the number of colonies in the treated samples compared with those in untreated controls.

Assay for ROS levels in cells. The intracellular ROS level was analysed by using 2',7'-dichlorofluorescein diacetate (DCFH-DA) following the method described by Kobayashi (2000) with some modifications. The non-fluorescent DCFH-DA was deacetylated by esterase within the cell to generate the polar compound 2',7'-dichlorofluorescein (DCFH), which is trapped in the cell. DCFH can be oxidized by ROS and converted into the fluorescent dichlorofluorescein (DCF). Cells were harvested by centrifugation at 5000 *g* for 10 min from 1 ml of the cell culture (OD₆₀₀ 1.0; 10⁷ cells ml⁻¹) and washed with phosphate buffer (PB; 50 mM, pH 7.5) and the precipitated cells were suspended in PB. An aliquot (50 μl) of the cell suspension was then incubated in 950 μl of 10 μM DCFH-DA at 37 °C for 30 min in the dark. During incubation, the suspension was mixed gently every 3–4 min. After incubation, the cells were harvested and washed twice in 5 ml PB to remove excess DCFH-DA which did not diffuse into the cell, and resuspended in 1 ml PB. Aliquots (500 μl) of the cell suspension were then incubated with 100 μl 30 mM H₂O₂ for 30 min (aliquot of untreated cells were used as a control) and 30 U catalase (Sigma) was added to stop the reaction. Excess catalase was removed by centrifugation and washing in PB. The fluorescence of DCF in cells was measured by a Shimadzu RF-5000 spectrofluorometer at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The level of ROS was represented

by the fluorescence of DCF. Stronger fluorescence indicated higher intracellular ROS levels. All tests were repeated four times.

Measurement of hydroxyl radical-scavenging activity of carotenoids. Hydroxyl radical-scavenging activity of carotenoids was assayed by the chemiluminescence method using the ABEL anti-oxidant test kit for hydroxyl radicals (Knight Scientific), following the manufacturer's instructions. Briefly, 25 µl of the carotenoid sample was mixed with 325 µl reconstitution and assay buffer and 50 µl Pholasin (a photoprotein that reacts with hydroxyl radicals to emit luminescence) in a cuvette. To start the reaction, the hydroxyl radical-generating reagents from the commercially available test kit were injected into the cuvette in the measuring chamber of a Sirius luminometer (Berthold Detection Systems) with a photon counter (370–630 nm). Luminescence was measured every second (expressed as counts s⁻¹). The amount of luminosity (total counts for 90 s) was calculated by integration. Scavenging activity (%) was calculated as $100 \times [(CL_{\text{control}} - CL_0) - (CL_{\text{sample}} - CL_0)] / (CL_{\text{control}} - CL_0)$, where CL_{control} is the luminosity of the control, CL_0 is the background luminosity and CL_{sample} is the luminosity of the tested sample.

Statistical analysis. The data were processed using Microcal Origin 6.0 (Microcal Software). Student's *t*-test was used to assess the significance of differences between results, and $P < 0.05$ was considered significant.

RESULTS

Identification of candidate gene and sequence analysis of CrtD homologue

By using comparative genome analysis of known carotenoid biosynthetic enzymes from other organisms, five *crt* gene homologues have been found in the genome of *D. radiodurans*, which encoded the following enzymes: CrtB

(phytoene synthase, DR0862) and CrtI (phytoene desaturases, DR0861) (Makarova *et al.*, 2001), CrtO (carotene ketolase, DR0093) (Tao & Cheng, 2004), CrtLm-type lycopene cyclase (DR0801) (Tao *et al.*, 2004) and a putative methoxyneurosporene dehydrogenase (DR2250). Fig. 2 shows a multiple sequence alignment of DR2250 with CrtD proteins and phytoene desaturases (CrtI) derived from photosynthetic and non-photosynthetic bacteria. DR2250 showed higher sequence identity to the carotenoid 3,4-dehydrogenases (CrtD) from *Rhodobacter capsulatus* and *Rubrivivax gelatinosus* (25.29 and 26.85 % identity, respectively), and showed lower sequence identity to the phytoene desaturases (CrtI) from *D. radiodurans* and *Pantoea ananatis* (21.24 and 21.89 % identity, respectively). Two conserved regions (underlined and marked 1 and 2) were observed in these carotenoid desaturases (Fig. 2). Conserved region 1 containing a GXGXXG motif at the N terminus was a putative dinucleotide- (ADP) binding domain (Bartley *et al.*, 1990). Conserved region 2 was a bacterial-type phytoene dehydrogenase signature with a pattern of (N/G)X(F/Y/W/V)(L/I/V/M/F)XG(A/G/C)(G/S)(T/A)(H/Q/T)PG(S/T/A/V)G(L/I/V/M)X₅(G/S) (data from PROSITE methods). The CrtD homologues from two *Deinococcus* species (DR2250_DEIRA and Dgeo2306_DEIGE) exhibited some distinguishing amino acids (marked with asterisks) from other protein sequences (Fig. 2).

Mutation of DR2250 and carotenoid analysis in the wild-type and mutant strains

Gene mutation of *dr2250* resulted in an orange strain, designated R1ΔCrtD, which was different from the red-

Fig. 2. Multiple sequence alignment of carotenoid desaturases from bacteria and cyanobacteria. DEIRA, *Deinococcus radiodurans* R1; DEIGE, *Deinococcus geothermalis* DSM 11300; RHOCA, *Rhodobacter capsulatus* SB1003; RHOS4, *Rhodobacter sphaeroides* ATCC 17023; THIRO, *Thiocapsa roseopersicina* BBS; RUBGE, *Rubrivivax gelatinosus* S1; MP993, marine bacterium P99-3; SYNY3, *Synechocystis* sp. strain PCC 6803; PANAN, *Pantoea ananatis* (*Erwinia uredovora*) 20D3; PARSN, *Paracoccus* sp. strain N81106/MBIC01143; STRGR, *Streptomyces griseus* JA3933. Identical and similar amino acids are denoted by black and grey backgrounds, respectively. Two conserved regions are underlined and numbered (regions 1 and 2): 1, conserved dinucleotide (ADP)-binding domain; 2, bacterial-type phytoene dehydrogenase signature. Amino acids that distinguish the sequences of DR2250_DEIRA and Dgeo2306_DEIGE from other protein sequences are marked by asterisks.

Fig. 3. Carotenoid analyses in *D. radiodurans* R1 wild-type and mutant strains. (a) Pigmentation of wild-type and mutant strains. (b–d) HPLC analysis of carotenoids from *D. radiodurans* R1 wild-type (b), mutant R1ΔcrtD (c) and mutant R1ΔcrtLm (d) at 470 nm. Peak 2, deinoxanthin; peak 6, lycopene. Other peaks are listed in Table 2.

pigmented wild-type strain (Fig. 3a). The carotenoids synthesized in mutant R1ΔcrtD were analysed by HPLC, and compared with those from the wild-type (Fig. 3b, c).

Peak 2 ($\lambda_{\text{max}}=452, 480$ and 507 nm in the HPLC eluent; $\lambda_{\text{max}}=453, 480$ and 507 nm in acetone; $\lambda_{\text{max}}=452, 480$ and 506 nm in ethanol, Fig. 4b and Supplementary Fig. S3) with a mass of 582 (Table 2) was identified as deinoxanthin (Lemee *et al.*, 1997; Saito *et al.*, 1998). Mutant R1ΔcrtD exhibited a similar HPLC profile to that of the wild-type strain, but its peaks were eluted slightly later than those of the wild-type. Moreover, all of the carotenoid peaks from mutant R1ΔcrtD demonstrated spectral blue shifts relative to the peaks of the wild-type strain (Fig. 4a–e), indicating that the carotenoid composition was altered by the mutation of *dr2250*. The mutant no longer produced deinoxanthin, but still accumulated a carotenoid (peak 2') with an obvious spectral blue shift ($\lambda_{\text{max}}=440, 468$ and 492 nm in the HPLC eluent; $\lambda_{\text{max}}=442, 468$ and 491 nm in acetone, $\lambda_{\text{max}}=441, 468$ and 491 nm in ethanol, Fig. 4b and Supplementary Fig. S4). The absorption spectrum of peak 2' did not show a fine structure. However, a fine structure appeared when peak 2' was reduced by NaBH₄ in ethanol ($\lambda_{\text{max}}=461$ nm; Supplementary Fig. S4d), indicating the presence of a conjugated keto group in peak 2'. From its main absorption maximum ($\lambda_{\text{max}}=468$ in methanol; Supplementary Fig. S4c), peak 2' was suggestive of 12 conjugated double bonds, including the conjugated keto group (Takaichi & Shimada, 1992; Takaichi, 2000). Deinoxanthin has 13 conjugated double bonds. These spectral properties suggested that the peak 2' compound was 3',4'-dihydrodeinoxanthin (Fig. 5). APCI mass analyses showed that the major carotenoid from mutant R1ΔcrtD (peak 2') with a mass of 584 (matching the formula of C₄₀H₅₆O₃) was compatible with 3',4'-dihydrodeinoxanthin (Table 2). The major carotenoid from mutant R1ΔcrtD (peak 2') is related to deinoxanthin but lacks the C-3',4' double bond, due to the lack of DR2250. The spectral blue shift of 3',4'-dihydrodeinoxanthin (peak 2') compared with deinoxanthin (peak 2) is a result of the reduced number of conjugated double bonds (Takaichi, 2000).

Peaks 3 and 4 had identical molecular masses (582) to that of deinoxanthin (Table 2). There was a characteristic *cis* peak ($\lambda_{\text{max}}=365$ nm) in the absorbance spectra of peaks 3 and 4 (Fig. 4c, d), which showed a %*D_B*/*D_H* value (the ratio of the peak height of the *cis* peak to that of the middle wavelength absorption band; Takaichi, 2000) of more than 30, compared with that of *trans*-deinoxanthin (less than 10). Peaks 3 and 4 were probably two *cis*-isomers of deinoxanthin. For the mutant R1ΔcrtD, peaks 3' and 4', with a molecular mass of 584, corresponded to the two *cis* forms of 3',4'-dihydrodeinoxanthin. The masses of peaks 3' and 4' were higher by 2 mass units than those of peaks 3 and 4 in the wild-type strain, indicating that these products synthesized in mutant R1ΔcrtD lacked one double bond.

The mass of peak 1 ($\lambda_{\text{max}}=452, 485$ and 507 nm in the HPLC eluent) and peak 1' ($\lambda_{\text{max}}=440, 468$ and 492 nm) (Fig. 4a) showed molecular ions at *m/z* 563 and 565 ([M–H][–]), matching the formulae C₄₀H₅₂O₂ and C₄₀H₅₄O₂, respectively (Table 2). They might be intermediate products in the carotenoid synthetic pathways of the wild-type and mutant strain, but their structures were not

Fig. 4. Absorption spectra of the carotenoids from wild-type and mutant strains. (a) Peak 1 ($\lambda_{\max}$ =452, 485 and 507 nm) and peak 1' ($\lambda_{\max}$ =440, 468 and 492 nm); (b) peak 2 (deinoxanthin; $\lambda_{\max}$ =452, 480 and 507 nm) and peak 2' (3',4'-dihydrodeinoxanthin; $\lambda_{\max}$ =440, 468 and 492 nm); (c) peak 3 ($\lambda_{\max}$ =365, 447, 475 and 503 nm; % D_B/D_{II} >30) and peak 3' (438, 466 and 489 nm; % D_B/D_{II} >30); (d) peak 4 ($\lambda_{\max}$ =365, 445, 473 and 502 nm; % D_B/D_{II} >30) and peak 4' ($\lambda_{\max}$ =364, 438, 466 and 489 nm; % D_B/D_{II} >30); (e) peak 5 ($\lambda_{\max}$ =455, 482 and 505 nm) and peak 5' ($\lambda_{\max}$ =440, 468 and 490 nm); (f) peak 6 (lycopene; $\lambda_{\max}$ =445, 473 and 504 nm; % III/II >60). Absorption spectra were measured in the HPLC eluent, acetonitrile/methanol/2-propanol (40:50:10, by vol.). Lycopene (Sigma) was used as a standard to identify peak 6. Peak numbers correspond to those in Fig. 3(b–d). Solid curves, wild-type; dotted curves, mutants. Absorbance zero is represented by the bottom of the vertical axis for all panels.

determined due to their low abundance. Peak 5 (m/z 565, $C_{40}H_{54}O_2$) from the wild-type and peak 5' (m/z 567, $C_{40}H_{56}O_2$) from the mutant were tentatively identified as 2-deoxydeinoxanthin and 2-deoxy-3',4'-dihydrodeinoxanthin, respectively, according to their absorbance and mass spectra (Fig. 4e; Table 2). The C-3',4'-desaturation products of lycopene or earlier intermediates were not detected in the wild-type or the mutant strain, suggesting that DR2250 can not catalyse desaturation of these acyclic substrates. CrtD in *D. radiodurans* may be a monocyclic carotenoid 3',4'-desaturase.

Mutation of DR0801 and carotenoid analysis of the mutant

Gene mutation of *dr0801* resulted in a pale red-pigmented strain and was designated R1ΔcrtLm (Fig. 3a). The

dominant carotenoid (peak 6 in Fig. 3d) in mutant R1ΔcrtLm showed the same absorption maxima as that of a lycopene standard (Fig. 4f). Peak 6 also showed the characteristic spectral fine structure of lycopene, with a % III/II of more than 60 (III/II refers to the ratio of the peak heights of the longest and the middle wavelength absorption bands from the minimum between the two peaks) (Takaichi, 2000). This component with a mass of 536 was identified as all-*trans*-lycopene (Table 2). The mutation of *dr0801* resulted in no synthesis of deinoxanthin but accumulation of lycopene. Therefore, DR0801 was confirmed to be lycopene cyclase (CrtLm) in the native host. No modified lycopene derivatives were detected in the wild-type or mutant strains. These results suggested that modification steps in the carotenoid biosynthesis of *D. radiodurans*, including hydroxylation, ketolation and C-3',4'-desaturation, are introduced after the cyclization of lycopene (Fig. 1).

Table 2. LC-mass spectroscopy analysis of selected carotenoids accumulated in the wild-type and mutants R1ΔcrtD and R1ΔcrtLm

Peaks 1–5 are from the wild-type strain, peaks 1'–5' are from mutant R1ΔcrtD and peak 6 is from mutant R1ΔcrtLm (as seen in Fig. 3b–d). ND, Not determined; intermediate products. The difference of one mass unit between the recorded masses $[M-H]^-$ and calculated molecular masses is due to deprotonation during mass analysis. Peaks 3 and 4 and 3' and 4' respectively represent *cis* isomers of deinoxanthin and 3',4'-dihydrodeinoxanthin.

Peak	Mass $[M-H]^-$ (<i>m/z</i>)	Calculated molecular mass	Matching formula	Tentative identification
1	563	564	C ₄₀ H ₅₂ O ₂	ND
1'	565	566	C ₄₀ H ₅₄ O ₂	ND
2	581	582	C ₄₀ H ₅₄ O ₃	Deinoxanthin
2'	583	584	C ₄₀ H ₅₆ O ₃	3',4'-Dihydrodeinoxanthin
3	581	582	C ₄₀ H ₅₄ O ₃	<i>cis</i> -Deinoxanthin
3'	583	584	C ₄₀ H ₅₆ O ₃	<i>cis</i> -3',4'-Dihydrodeinoxanthin
4	581	582	C ₄₀ H ₅₄ O ₃	<i>cis</i> -Deinoxanthin
4'	583	584	C ₄₀ H ₅₆ O ₃	<i>cis</i> -3',4'-Dihydrodeinoxanthin
5	565	566	C ₄₀ H ₅₄ O ₂	2-Deoxydeinoxanthin
5'	567	568	C ₄₀ H ₅₆ O ₂	2-Deoxy-3',4'-dihydrodeinoxanthin
6	535	536	C ₄₀ H ₅₆	Lycopene

Survival rates and intracellular ROS levels in the wild-type and the mutant deficient in CrtD under stress

Fig. 6(a) shows that the mutant R1ΔcrtD became more sensitive to oxidative stress than the wild-type strain. The survival rate of the mutant decreased significantly with increasing concentrations of H₂O₂, illustrating that CrtD contributes to the antioxidant capacity of deinoxanthin in *D. radiodurans*. The total cellular carotenoid level in the mutant R1ΔcrtD [$57.8 \pm 2.1 \mu\text{g}$ (g dry cell weight)⁻¹] was similar to that in the wild-type [$55.2 \pm 1.5 \mu\text{g}$ (g dry cell weight)⁻¹] ($P > 0.05$, not significant), demonstrating that the difference of survival rates between the mutant and the wild-type strain was not caused by the total cellular carotenoid levels.

External H₂O₂ can penetrate the cytoplasmic membrane to form other intracellular ROS such as singlet oxygen by the Mallet reaction (Deby-Dupont *et al.*, 1998) and the most

Fig. 5. Structures of major carotenoids identified from the wild-type and mutant strains: deinoxanthin (peak 2 in the wild-type), 3',4'-dihydrodeinoxanthin (peak 2' in mutant R1ΔcrtD) and lycopene (peak 6 in mutant R1ΔcrtLm).

Fig. 6. (a) Survival curves for the wild-type (○) and mutant R1ΔcrtD (●) following H₂O₂ treatment. (b) Intracellular ROS level in cells of the wild-type and mutant R1ΔcrtD in the presence (hatched bars) and absence (open bars) of 30 mM H₂O₂. Each point represents the mean $\pm$ SD of four independent experiments.

harmful hydroxyl radical by the Fenton reaction (Imlay, 2003). Therefore, the ROS level in the wild-type and mutant R1ΔcrtD cells with or without H₂O₂ treatment was further measured. As shown in Fig. 6(b), mutant as well as wild-type cells showed little ROS in the cell in the absence of H₂O₂ (control); however, the intracellular ROS level in both the wild-type and the mutant increased remarkably in the presence of H₂O₂. The mutant cell generated more ROS than the wild-type when exposed to H₂O₂, showing that the mutation of *crtD* resulted in decreased cell resistance to ROS. The loss of the C-3',4' double bond may have lowered the antioxidant capacity of carotenoids in *D. radiodurans*.

Comparison of the antioxidant capacity of the carotenoid product of R1ΔcrtD with that of the wild-type

The major product from mutant R1ΔcrtD, 3',4'-dihydrodeinoxanthin, which lacks the double bond at C-3',4', exhibited a significantly decreased hydroxyl radical-scavenging activity compared with deinoxanthin ($P<0.05$) (Fig. 7). Both deinoxanthin and 3',4'-dihydrodeinoxanthin showed higher scavenging activities than astaxanthin, a xanthophyll with hydroxyl group and keto end-group substitutions.

DISCUSSION

On the basis of investigations of the *crt* gene homologues of *D. radiodurans* in this work and in previous studies (Tao & Cheng, 2004; Tao *et al.*, 2004; Tian *et al.*, 2007; Xu *et al.*, 2007; Zhang *et al.*, 2007), the deinoxanthin biosynthetic pathway is proposed as shown in Fig. 1. DR0862 was identified to be CrtB (phytoene synthase), which catalyses the synthesis of phytoene (Tian *et al.*, 2007; Zhang *et al.*, 2007), the first committed step in the carotenoid biosynthetic pathway of

Fig. 7. Relative scavenging activities (hydroxyl radicals) of deinoxanthin (■), the major carotenoid from mutant R1ΔcrtD (●) and astaxanthin (□). Values are means $\pm$ SD of three experiments. Concentration (mM) refers to the concentration of each carotenoid.

bacteria. The gene *dr0861* was identified to encode the phytoene desaturase, which transforms phytoene into all-*trans*-lycopene (Xu *et al.*, 2007; Zhang *et al.*, 2007). The expressed CrtLm homologue (DR0801) can convert lycopene to γ -carotene in a lycopene-accumulated *Escherichia coli* strain (Tao *et al.*, 2004), but its catalysing step in the native host has not been verified.

Mutation of the putative *crtD* gene (*dr2250*) and carotenoid analysis of the mutant confirmed that *dr2250* encodes carotenoid 3',4'-desaturase. Analyses of other carotenoid intermediates from the mutant also provided evidence for the C-3',4'-desaturase activity of DR2250. C-3',4'-desaturation products of lycopene or earlier intermediates were not detected in the wild-type or any mutant, suggesting that DR2250 can not act on these acyclic substrates. Therefore, CrtD in *D. radiodurans* is a monocyclic carotenoid 3',4'-desaturase which catalyses the desaturation of 3',4'-dihydrodeinoxanthin to yield deinoxanthin, and thus differs from CrtD known in purple bacteria (*Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rubrivivax gelatinosus* and *Thiocapsa roseopersicina*; Bartley *et al.*, 1990; Lang *et al.*, 1995; Steiger *et al.*, 2000; Kovács *et al.*, 2003) and an aerobic photosynthetic bacterium (*Bradyrhizobium* ORS278; Giraud *et al.*, 2004). CrtD from these bacteria catalyses the introduction of C-3,4 double bonds into acyclic carotenoids. Slr1293 from *Synechocystis* sp. strain PCC 6803 was reported to have the function of CrtD, and used neurosporene as its substrate to form 3',4'-didehydroneurosporene and was essential for the myxoxanthophyll biosynthesis (Mohamed & Vermaas, 2004). Recently, Maresca *et al.* (2008) reported that an independently constructed Slr1293 mutant still synthesized myxoxanthophyll. In a lycopene-accumulating *E. coli* strain expressing pAC-LYC or a γ -carotene-accumulating *E. coli* strain expressing pAC-GAMMA (kindly provided by F. X. Cunningham, University of Maryland) (Cunningham *et al.*, 2007), the introduction of *dr2250* resulted in no newly formed carotenoids (data not shown). This suggested that lycopene and γ -carotene are not substrates for DR2250. It was reported that CrtD from *Rubrivivax gelatinosus* required a hydroxyl group at position C-1 for C-3,4 desaturation (Steiger *et al.*, 2000). The carotenoid components of mutant R1ΔcrtD had hydroxyl group substitution, indicating that the C-3',4'-desaturation reaction also needed hydroxylation modification in *D. radiodurans*. The CrtD from the marine bacterium strain P99-3 can use 1'-hydroxy- γ -carotene as its substrate to form 1'-hydroxy-torulene in *Escherichia coli* with P99-3 CrtD (Teramoto *et al.*, 2004). However, DR2250 catalysed the desaturation of 3',4'-dihydrodeinoxanthin, a monocyclic substrate with ketolation and hydroxylation on the β -ring, to yield deinoxanthin in the native host.

The mutant R1ΔcrtLm no longer synthesized deinoxanthin but accumulated only lycopene, confirming that *dr0801* encodes lycopene cyclase (Fig. 1). The substituents on the end group including ketolation, C-2 hydroxylation, C-1',2' hydration and C-3',4' desaturation are therefore added after the cyclization of lycopene. Other carotenogenic enzymes in the proposed pathway not yet identified in *D.*

radiodurans are carotenoid 1',2'-hydratase (CrtC) and 2-hydroxylase. CrtC has previously been identified in some bacteria (Armstrong *et al.*, 1989; Ouchane *et al.*, 1997; Maresca & Bryant, 2006; Giraud *et al.*, 2004). Recently, a carotenoid 2,2'- β -hydroxylase (CrtG) was detected in *Brevundimonas* sp. strain SD212 (Nishida *et al.*, 2005). However, no homologues of these genes have been found in the genome of *D. radiodurans*.

Cell resistance to oxidant stress was affected by *crtD* mutation. The mutant was more sensitive to oxidant stress than the wild-type strain (Fig. 6), illustrating that CrtD contributes to the antioxidant activity of carotenoids in *D. radiodurans*. 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). We demonstrated previously that deinoxanthin is a stronger scavenger of ROS than carotenes (lycopene and β -carotene) or xanthophylls (zeaxanthin and lutein) (Tian *et al.*, 2007). The carotenoids in *D. radiodurans* provided protection to the cell against oxidative stress, although the effect is not so remarkable as the effect of DNA-repair proteins. As non-enzymic ROS scavengers, carotenoids in *D. radiodurans* may act together with PQQ (Misra *et al.*, 2004) and a high intracellular Mn(II) to Fe(II) ratio (Daly *et al.*, 2004, 2007) in intracellular resistance to ROS. Carotenoids and PQQ in *D. radiodurans* can scavenge the hydroxyl radical in particular, which can not be eliminated directly by catalase or superoxide dismutase. Carotenoids might also contribute to the resistance of the cell by inhibiting oxidative damage to proteins and inhibiting lipid peroxidation of the membrane (Maresca & Bryant, 2006).

The present study on the role of carotenoid 3',4'-desaturase (CrtD) in *D. radiodurans* highlights the possible reaction sequence involved in deinoxanthin biosynthesis. C-3',4' desaturation contributes to the antioxidant activity of deinoxanthin. Further investigations on the remaining unidentified genes including carotenoid 1',2'-hydratase and carotenoid 2-hydroxylase are needed to elucidate the carotenoid biosynthetic pathway in *D. radiodurans*.

ACKNOWLEDGEMENTS

This work was supported by the National Basic Research Program of China (2004CB19604, 2007CB707804), the National Hi-Tech Development Program (2007AA021305), the National Natural Science Foundation of China (grant no. 30670026), the Distinguished Young Scientists Program of China (30425038), the Zhejiang Provincial Natural Science Foundation of China (grant no. Y306075) and the Science and Technology Department of Zhejiang Province of China (2006E10058).

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Edited by: J Green